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(54) Title: GENE THERAPY FOR TRANSPLANTATION AND INFLAMMATORY OR THROMBOTIC CONDITIONS

(57) Abstract

A method to render in particular endothelial cells capable of inhibiting platelet and leukocyte-mediated injury and inflammation is described, comprising genetically modifying the cells by inserting therein DNA encoding ATP diphosphohydrolase or an oxidation-resistant analog thereof, and expressing a protein having functional ATP diphosphodydrolase activity, such as the human CD39 protein, from the cells under cellular activating conditions, and corresponding cells, tissue or organs, non-human transgenic or somatic recombinant mammals, pharmaceutical compositions and prosthetic intravascular devices. The invention, which can be carried out *in vivo*, *ex vivo* or *in vitro*, has use in allogeneic or xenogeneic transplantation as well as in treating systemic or local inflammatory conditions characterized by platelet aggregation leading to thrombus formation.

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GENE THERAPY FOR TRANSPLANTATION AND INFLAMMATORY OR THROMBOTIC CONDITIONS

Field of the Invention

The invention provides improvements in the field of gene therapy and tissue and organ transplantation. In its broad aspect it is concerned with genetic modification of endothelial cells to render such cells less suceptible to an inflammatory or other activating stimulus.

In particular, the invention concerns genetic modification of endothelial cells subject to a platelet-mediated activation stimulus, to render them capable of inhibiting platelet aggregation by expressing functional ATP diphosphohydrolase activity under conditions of endothelial cell activation or inflammation.

In a preferred embodiment, the invention is addressed to a novel use of the polypeptide or class of polypeptides previously identified as a B cell activation marker, CD39. It has now been found that CD39, a cell surface glycoprotein associated with B lymphocytes, activated NK cells, certain T cell and endothelial cells, but heretofore unassigned a cell-specific function, exerts an ATP- and ADP-degrading, i.e. ATP-diphosphohydrolase, activity. The novel use of CD39 which is contemplated by this invention therefore comprises the suppression or inhibition of ADP-induced platelet aggregation and thrombus formation, particularly under cellular activating conditions or in connection with tissue inflammation. Accordingly, the invention in its further aspects and embodiments is concerned with genetic modification of mammalian cells, and tissues or organs comprising said cells, to render such cells, organs or tissues capable of expressing CD39 protein, and maintaining the function of expressed protein at sufficient levels under cellular activating conditions, whereby platelet aggregation at the surface of said cells (and, ultimately, thrombus formation) are suppressed or inhibited.

The invention also contemplates use of CD39 protein or gene coding therefor in connection with such further embodiments as are disclosed herein in general for an ATP diphosphohydrolase active protein.

Background of the Invention

Thromboembolic phenomena are involved in a number of vascular diseases and pathologies, including a variety of atherosclerotic and thrombotic conditions, for example, acute myocardial infarction, chronic unstable angina, transient cerebral ischemic attacks and strokes, carotid endarterectomy, peripheral vascular disease, restenosis, and/or thrombosis following angioplasty, or anastomosis of cardiovascular devices, such as catheters or shunts. Also relevant are preeclampsia, as well as various forms of vasculitis, e.g. Takayasa's disease and rheumatoid vasculitis. Of importance is that in the field of allogeneic or xenogeneic transplantation, thrombus formation in the vasculature of grafts is a serious problem affecting the viability of implanted tissues and organs.

A recognized component of the body's complex physiological mechanism for generating a thrombus is the sequence of events giving rise to platelet activation (also referred to as platelet "adhesion" and "aggregation"). In brief, the endothelium (also known as the "vascular endothelium") consists of a layer of cells that line the cavities of the heart and of the blood and lymph vessels. The process of "activation" of endothelial cells by platelet and leukocyte mediated injury and inflammation, with accompanying release of activating agents, such as the cytokine TNFα, has been described in the literature, *see* F. Bach et al., Immunological Reviews 141 (1994) 5-30 and Pober and Cotran, Transplantation 52 (1991) 1037-1042. A phenomenon associated with this process is the retraction of the endothelial surface and exposure of constituents of the subendothelial matrix, such as collagen and von Willebrand Factor (vWF).

Concomitantly with endothelial "activation", the platelets, normally freely circulating in the blood, become "activated" by the exposed constituents of the subendothelial matrix, as well as by thrombin and activated complement components. In this activated state, enhanced expression of platelet glycoprotein (GP)IIb/IIIa and P-selectin promotes affinity for components of the endothelium and subendothelium. Additionally, platelets begin to secrete biologically active constituents, in particular, the adenine nucleotides, ATP and ADP. ADP is essential for continued platelet activation response and leads to further recruitment of platelets. ATP also stimulates neutrophils via their P2y receptors and results in the increased release of reactive oxygen intermediates. In a continuing inter-related sequence of events, platelet "aggregation" is initiated by the binding of agonists such as ADP, as well as thrombin, epinephrine, ADP, collagen and thromboxane A2, to platelet membrane receptors. Stimulation by agonists results in exposure of latent fibrinogen receptors on the platelet surface, and finally, the binding of fibrinogen to the platelet GPIIb/IIIa receptor complex, which is believed to be principally responsible for platelet aggregation and thrombus formation in vivo.

Opposing the above-described platelet aggregation process are various potent antithrombotic mechanisms which are primarily localized to the endothelium, e.g. (i) release release of prostacyclines, (ii) generation of nitric oxide, and (iii) activity of ADP-degrading enzymes, and fibrinolytic mechanisms. However, it is self-evident that these mechanisms may be ineffective and are unable to prevent many inflammatory vascular disorders, or to maintain graft survival, with the result that platelet activation and aggregation proceed, largely unregulated, to ultimate vascular occlusion and platelet thrombosis.

Graft injury and loss seen with graft preservation-induced endothelial damage, as well as in allograft and xenograft rejection, exemplify the vulnerability of endothelial tissue in the activated condition to thrombotic complications. For example, following anastomosis of the

vasculature of a graft, recipient platelets begin to interact with endothelial and subendothelial cells of the graft. Activation of the graft endothelium in an inflammatory environment can initiate the platelet aggegation cascade, with consequent adhesion and aggregation of the platelets on the graft endothelium, rendering the graft susceptible to thrombosis and, ultimately, graft failure.

Considerable effort by workers in the art has been directed toward elucidation of agents which can control platelet aggregation. However, antiplatelet agents currently in clinical use have recognized side-effects, and suffer lack of selectivity. Newer GPIIb/IIIa antagonists, such as peptides, peptidomimetics and antibodies are more selective and potent but do not serve a prophylactic function in the early stages of inflammation or injury. Certain purinergic P2T receptor antagonists, and to some extent PAF antagonists, have similar shortcomings. Thus there exists a critical need for a method to prevent or minimize platelet aggregation occurring in connection with endothelial cell activation. In particular, there is a need to prolong graft organ survival, while minimizing toxicity and other adverse effects associated with available platelet activation inhibitors.

Summary of the Invention

It has now been found that regulation and inhibition of platelet aggregation under cellular activating conditions are critically dependent on the maintenance of an ecto ATP-diphospho-hydrolase activity by endothelial cells. More particularly, it has been found that activation of endothelial cells (hereinafter "EC") in response to an immune or inflammatory stimulus leads to the reduction or loss of the ADP-hydrolyzing activity on the surface of said cells; and furthermore, this reduction or loss of ADP-hydrolyzing activity results in platelet adhesion to the endothelial cell surface and plat let aggregation, and ultimately 1 ads to thrombus formation.

In particular, it has been observed that EC, in the absence of activating agents, can express a cell-associated ATP-diphosphohydrolase activity which is capable of inhibiting platelet activation, and that under conditions promoting activation of EC (e.g. exposure to TNFα/complement and hyperacute rejection of a xenograft/ reperfusion injury/oxidative stress), there is a reduction or loss of ecto ATP-diphosphohydrolase activity, resulting in a cellular environment with increased susceptibility to platelet aggregation.

It has further been found that the activity of native mammalian/porcine ATP diphosphohydrolases is suceptible to oxidation, and when oxidized, the protein loses the ability to suppress platelet activation. It is now believed that this phenomenon plays a significant role in many pathogenic states, including platelet aggregation and thrombus formation seen with graft rejection. Many of the pathologies or disease conditions requiring therapy directed toward suppressing platelet aggregation are associated with high levels of toxic oxygen radicals and other reactive oxygen intermediates. An example of such a pathology is graft preservation injury and ischemia- reperfusion. Implicated disease states are reperfusion injury associated with myocardial infarction, disseminated intravascular coagulation associated with septicemia, alveolar fibrosis associated with adult respiratory syndrome, and noncardiogenic pulmonary edema. Furthermore, injury to the endothelium involves the influx of activated monocytes, polymorphonuclear leukocytes, etc., which can also create toxic oxygen species.

While hitherto a general connection between endothelial cell damage, inflammation and thrombosis had been recognized, it has been established first with the present invention that the enzyme ATP diphosphohydrolase, under conditions of oxidant stress, exhibits diminished ability to prevent platelet aggregation. This novel feature is critically important in the treatment of many of the pathological conditions requiring restoration of a cellular plat let activation-suppressing, or

anti-thrombotic function.

It has now also been found that significant, e.g. 95% or greater, typically 98% or greater, e.g., 99% and greater, and even 100%) homology exists between peptide sequences corresponding to type I and type II ecto-ATP diphospho-hydrolases, such as reported by Christoforidis et al., Eur. J. Biochem. 234(1) (November 15, 1995) 66-74, and the CD39 lymphocyte activation marker [C.R. Maliszewski et al., J. Immunol. 153 (1994) 3574-3583]. It had been previously unappreciated in the art that the CD39 protein or class of proteins encodes an ATP hydrolyzing function, in particular an ecto-ATP diphosphohydrolase.

Therefore, the term "ATP diphosphohydrolase" or "ecto-ATP diphosphohydrolase" refers to and includes native CD39 protein (especially, native human CD39 protein).

Accordingly, the invention in its broader aspects concerns a **method of genetically-modifying mammalian, e.g. endothelial cells** to render them less susceptible to an inflammatory or immunological stimulus and platelet adhesion, which comprises conferring on such cells the capability of stably expressing a polypeptide having activity of an ATP diphosphohydrolase under cellular activating conditions, i.e. of expressing ATP diphosphohydrolase at levels sufficient to suppress or inhibit platelet adhesion or aggregation at the cell surface.

By "stably" expressing is meant that transcription and expression of the ATP diphosphohydrolase protein or analog thereof by the cell is maintained at antithrombotic (i.e. platelet plug/thrombosis-suppressing) effective amounts. Such concentrations of the protein may be the same, higher or even lower than is expressed by the cell under hemostatic conditions; however, such "stable" expression of the ATP diphosphohydrolase protein is sufficient to result in a reduction or suppression of platelet aggregation and platelet thrombi in the vasculature in the local micro-environment of the cell, i.e. at the surface of the modified cell, as

compared to a cell under similar activation conditions which is not modified according to the invention, i.e. does not contain the inserted gene/protein.

By "cellular activation conditions" is meant Type I EC activation (referring to early events following stimulation, which include the retraction of EC from one another as well as hemorrhage and edema); and/or Type II EC activation (referring to later events which occur over hours and are dependent upon transcriptional regulation and protein synthesis) (see Bach et al., supra). A generally accepted indicator of Type I EC activation is an elevated level of PAF and/or P-selectin in the cellular environment. A generally accepted indicator of Type II EC activation is an elevated level of E-selectin in the cellular environment or membranes.

Suppression or inhibition of platelet adhesion or aggregation at the surface of a cell modified according to the invention can be determined by known methods, e.g. as described in Marcus et al., J.Clin.Investig. 88 (1988) 1690-1696 and Born, Nature 194 (1962) 927-930 [reviewed in Peerschke, Semin.Hematol. 22 (1985) 241]. A reduction in platelet aggregate formation at the surface of the cell of 50% and greater, and preferably 65% and greater, demonstrates platelet inhibition or suppression for purposes of the invention.

The stable, or high-level, ADP-hydrolyzing activity provided by the invention can be obtained using **vector constructs** comprising DNA encoding a polypeptide having ATP-diphosphohydrolase activity, in particular ATP diphosphohydrolase protein, under the control of a promoter capable of initiating transcription of the DNA under conditions of cell activation or oxidative stress, and thus replace the activity of the normally present ATP diphosphohydrolase. Examples of such promoters include "constitutive" or "inducible" promoters.

By "constitutive" is meant that protein expression is essentially independent of cellular activation factors, and is essentially continuous over the life of the cell.

By "inducible" is meant that protein expression can be controlled by administration of exogenous factors either not typically present in the cellular environment, or lost or diminished from the cellular environment under activating conditions. Such exogenous factors may include cytokines or growth factors.

It is also within the scope of the invention to achieve "stable" ATP-diphosphohydrolase activity by providing peptides that have ADP-hydrolyzing activity under oxidizing conditions. Thus the invention provides **peptide analogs** having activity of a native ATP-diphosphohydrolase such as CD39, preferably human CD39 protein, and which are substantially oxidation-resistant.

Also contemplated is co-administration of an anti-oxidant to the affected cell, tissue or organ, concomitantly with expression of the ecto-ATP diphosphohydrolase.

Accordingly, the invention in its more particular aspects comprises a **method of genetically modifying mammalian,** e.g. endothelial **cells** and monocytes, NK cells, lymphocytes, red blood cells and islet cells to render them capable of inhibiting platelet aggregation, which comprises: inserting into the cells, or progenitors thereof, DNA encoding a polypeptide having activity of an ATP diphosphohydrolase, especially encoding functional ecto-ATP diphosphohydrolase protein, or an oxidation-resistant analog thereof, particularly in operative association with an inducible promoter, and expressing such polypeptide, particularly ecto-ATP diphosphohydrolase from the cells under cellular activating conditions at platelet aggregation, suppressing effective levels.

By "functional" is meant that the expressed ATP-diphosphohydrolase of such cells hydrolyzes platelet-secreted ADP to AMP and monophosphate.

The invention also comprises **a method of controlling platelet aggregation and thereby preventing or alleviating a thrombotic condition in a mammalian subject in need of such**

therapy, comprising genetically modifying cells, preferably endothelial cells, of the subject susceptible to platelet-mediated activation by inserting therein DNA encoding a polypeptide having ATP diphosphohydrolase activity or an oxidation-resistant analog thereof, particularly in operative association with a suitable promoter, and expressing the polypeptide from such cells at platelet aggregation-suppressing effective levels. Preferably the cells are modified in vivo, i.e. while remaining in the body of the subject.

In another aspect, cell populations can be removed from the patient, genetically modified ex vivo by insertion of vector DNA, and then re-implanted into the subject. The subject is preferably human.

In a further aspect the invention includes a **method of transplanting donor allogeneic or xenogeneic cells, preferably endothelial cells, or graftable tissue or organs comprising such cells**, to a mammalian recipient in whose blood or plasma these cells or tissue or organs are susceptible to an activation stimulus, which comprises:

(a) genetically modifying such donor cells, or progenitor cells thereof, by inserting therein DNA encoding a polypeptide having activity of an ATP-diphosphohydrolase or an oxidation-resistant analog thereof in operative association with a promoter; and

(b) transplanting the resultant modified donor cells, tissue or organs into the recipient and expressing from the resultant modified cells or tissue or organs the polypeptide having ATP diphosphohydrolase activity at platelet-aggregation suppressing effective levels.

The "modified donor cells" of step (b) refer to cells which themselves were subject to genetic modification in step (a), as well as to progeny cells thereof. These also form part of the invention.

Steps (a) and (b) may be carried out in either order; namely, the above donor allogeneic or xenogeneic cells, tissue or organs, may be modified or genetically engineered (e.g. by

transfection, transduction or transformation) prior to, or alternatively after, implantation into the recipient.

For example, endothelial cells from tissue or organs of a pig may be genetically modified in vivo by insertion of DNA encoding human ATP-diphosphohydrolase protein or an oxidation-resistant analog thereof under the control of a promoter, and the modified cells or tissue are then recruited for grafting into a human recipient. Once transplanted, the transgenic cells or tissue or organs express functional human ecto-ATP-diphosphohydrolase or an oxidation-resistant analog thereof, even in the presence of otherwise down-regulatory factors and in an inflammatory environment.

Since porcine or bovine ATP-diphosphohydrolase factors, for example, have cross-species activity, porcine or bovine protein-expressing transgenic (or somatic recombinant) animals may usefully be employed for recruitment of cells, tissues and organs for transplantation to humans. Preferably, however, the human protein or analog in a suitable vector will be used to modify porcine donor cells or organs to render them transgenic (or somatic recombinant) for transplantation purposes.

Somatic recombinant or transgenic donor animals can be obtained by modifying cells of the animal, or earlier, e.g. at the embryonic stage, by well-known techniques, so as to produce an animal expressing the desired protein.

Donor cells or tissue can also be genetically modified ex vivo, whereby cells, tissues or organs extracted from the donor and maintained in culture are genetically modified as described above, and then transplanted to the recipient, where the graft can then express the desired functional protein.

It is preferred that the genetic modification of the donor be done in vivo.

According to a further aspect of the invention, there are provided **cells, particularly endothelial cells, or tissue or organs** of a donor mammalian species, the cells, tissue or organs being modifi d to be capable of expressing DNA encoding a polypeptide having ATP-diphosphohydrolase activity at platelet-suppressing effective levels in a graft recipient of the same or a different sp cies as the donor under cellular

activating conditions.

The invention further provides a **non-human transgenic or somatic recombinant mammal** comprising in its cells, particularly its endothelial cells, heterologous DNA encoding a polypeptide having activity of an ATP-diphosphohydrolase, under cellular activating conditions, and such **cells, tissue and organs** per se; and a **method of preparing such non-human transgenic or somatic recombinant mammal**. Such non-human transgenic or somatic recombinant animals are particularly of the porcine species; murine transgenics expressing human ATP diphosphohydrolase are however also within the scope of the invention.

Also included is a **method of inhibiting platelet-aggregation and thereby treating thrombotic disorders in a mammalian (e.g. human) subject**, comprising administering to the subject an amount effective for inhibiting platelet aggregation of a recombinant polypeptide having ATP-diphosphohydrolase activity or pharmaceutically acceptable salt thereof, or an oxidation-resistant analog thereof, and **pharmaceutical compositions** comprising such polypeptide or pharmaceutically acceptable salt thereof, or an oxidation-resistant analog thereof, preferably in soluble form, in a pharmaceutically acceptable carrier.

Also contemplated are **prosthetic intravascular devices** comprising a synthetic biocompatible material having applied thereto recombinant ATP-diphosphohydrolase or an oxidation-resistant analog thereof as defined above.

Such therapies are useful to alleviate thrombotic conditions in a patient, and in particular to moderate thrombotic complications occurring in connection with organ transplantation, especially where the graft recipient is human. The invention further includes the **use of a recombinant polypeptide having ATP diphosphohydrolase activity** or pharmaceutically acceptable salt thereof, or an oxidation-resistant analog thereof, especially human CD39 protein, in the preparation of a medicament for reducing platelet aggregation, in particular in thrombosis.

Description of the drawings

Fig. 1: **Modulati n of cto-ADPase**: Bar graph depicting the inhibitory effect of human rTNFα on ecto-ATP diphosphohydrolase activity:
■ = TLC nmol ADP/million cells/min;
□ = LeBel/Fiske µmol phosphate/hr/mg cell protein [Example 1(c)].rTNFα = recombinant tumor necrosis factor α.

Fig. 2: **LWB (Lineweaver Burke) ectoADPase** (a double reciprocal plot of enzyme kinetics): This depicts the kinetics of quiescent and cytokine-mediated PAEC:
■ = control; □ = TNF
[Example 1(d)].

Fig. 3: **Inhibition of ectoADPase activity by oxidative stress and cellular activation (HOOH 5 µM/ectoAdPase)**: Bar graph depicting peroxide and cytokine mediated loss of ecto-ATP diphosphohydrolase activity on PAEC
[Example 2(a)].

Fig. 4: **Protective effects of β-mercaptoethanol on ectoADPase activity**: Bar graph demonstrating that β-mercaptoethanol (BME) protects against cytokine-mediated loss of ecto-ATP diphosphohydrolase activity on PAEC
[Example 2(b)]. BME = β-mercaptoethanol.

Fig. 5: **Kinetics of ectoADPase modulation**: Bar graph showing kinetics of ecto-ATP diphosphohydrolase modulation by TNFα and oxidants: ■ = control;
□ = XO/X; ■ = HOOH; ■ = TNF (in that order on the graph)
[Example 2(c)]. XO/X = xanthine oxidase/xanthine; HOOH = hydrogen peroxide.

Fig. 6: **Modulation of ectoADPase activity by antioxidants:** Plot of ecto-ATP diphosphohydrolase activity of activated PAEC treated with antioxidants [Example 3]. SOD = superoxide dismutase; Cat = catalase.

Fig. 7: **Reperfusion injury:** Bar graph showing ecto-ATP diphosphohydrolase activity in purified rat glomeruli as a function of reperfusion time in vivo [Example 5]. Isch = ischaemic time (min); Reperf = reperfusion time (min).

Fig. 8: **Effect of CVF:** Bar graph demonstrating effect of pre-treatment with cobra venom factor (CVF) of rat glomeruli rendered ischaemic and then reperfused [Example 6].

Fig. 9: **Northern analysis of CD39:** HUVEC following TNFα stimulation show diminished levels of mRNA for CD39 [Example 7]. hEC = HUVEC = human umbilical vein endothelial cells; TNF = recombinant tumor necrosis factor.

Fig. 10: **Transient transfection of COS-7 cells with pCDNA3/CD39:** FACS analysis of non-transfected COS-7 cells and COS-7 cells transfected with CD39 cDNA. Analysis by moAB (= monoclonal antibody) to CD39. Isotype control used concurrently. Cells were stained with moAB (Accurate) to human CD39.

Fig. 11: **EctoADPase activity of CD39-transfected COS-7 cells:** Whole cell lysate of COS-7 cells transfected with CD39 cDNA express specific Ca^{++}-dependent ecto-ADPase activity (substrate = 200 µM ADP). First bar: control; second bar: empty vector; third bar: CD39 vector.

Fig. 12: EctoADPase activity of purified membranes of COS-7 cells transfected with CD39: Activity localized primarily to cell membranes. First bar: control COS cells; second bar: COS cells transfected with empty vector; third bar: COS cells transfected with CD39 vector.

Fig. 13: Platelet aggregation assay: Inhibition of platelet aggregation by CD39; aggregation of PRP with 5 µM ADP and COS-7 cell membrane extracts (27.4 µg protein). COS-7 cell membrane extracts from CD39-transfected cells effectively inhibit platelet aggregation induced by ADP 5 µM, confirming the functional potential of the CD39/ectoADPase protein.

Fig. 14: Human CD39 nucleotide and amino acid sequence (from J.Immunol. 153 (8) [1994] 3577) (= **SEQ ID No.1**).

Definitions

"Graft," "transplant" or "implant" are used interchangeably to refer to biological material derived from a donor for transplantation into a recipient, and to the act of placing such biological material in the recipient.

"Host or "recipient" refers to the body of the patient in whom donor biological material is grafted.

"Allogeneic" refers to the donor and recipient being of the same species. As a subset thereof, "syngeneic" refers to the condition wherein donor and recipient are genetically identical. "Autologous" refers to donor and recipient being the same individual. "Xenogeneic" and "xenograft" refer to the condition where the graft donor and recipient are of different species.

"ATP diphosphohydrolase": an enzyme capable of catalyzing the sequentual hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) to adenosine

monophosphate (AMP) (the enzyme is also alternately referred to as ADPase; ATPDase; ATPase; ADP monophosphatase; or apyrase; EC 3.6.1.5).

The term "a polypeptide having activity of an ATP diphosphohydrolase" includes native ecto-ATP diphosphohydrolase protein, as well as oxidation resistant peptide analogs thereof, and soluble truncated forms.

An example of an ecto-ATP diphosphohydrolase is the CD39 protein. "CD39" refers to a natural mammalian gene (including cDNA thereof) or protein, including derivatives thereof having variations in DNA or amino acid sequence (such as silent mutations or deletions of e.g. up to 5 amino acids) which do not prejudice the ATP-hydrolyzing activity of the protein. The CD39 gene or protein employed in the invention may, for example, be porcine, bovine or human, or may be of a primate other than a human, depending on the nature of the cells to be modified and, for example, the intended recipient species for transplantation. The term "human CD39" as used herein refers to a protein which is at least 70%, preferably at least 80%, more preferably at least 90% (e.g., 95% or greater, e.g. 99% or 100%) homologous to the amino acid sequence of the CD39 lymphocyte activation marker cloned from a human B cell lymphoblastoid cell line by C.R. Maliszewski et al. (Genbank/NCBI accession number 765256; 23 March 1995) in J. Immunol. 153 (8) (1994) 3574-3584 [**SEQ ID No.1**].

Detailed Description of the invention

The **ATP diphosphohydrolases** comprise a family of proteins which catalyze the sequential phosphorolysis (i.e. removal of phosphate groups) of ATP to ADP to AMP. In general, proteins of this class exhibit nonspecificity toward nucleoside di- or triphosphates; and are activated by Ca^{2+} or Mg^{2+}. By converting ADP into AMP, as well as ATP, via ADP, into AMP, these enzymes inhibit or reverse platelet aggregation. The final product, AMP, is a substrate for 5' nucleotidases and generates adenosine, an important platelet anti-activator and vasodilator.

The proteins are primarily found in the cellular elements of the blood and the vascular wall. For such cellular enzymes to be effective, the enzymes should be functional at the cell surface, i.e. as ecto-enzymes. Because the ATP diphosphohydrolases are membrane-associated, insoluble proteins expressed on the cell surface, they are conventionally referred to as ecto-ATP diphosphohydrolases. **Soluble analogs** of such proteins may also be prepared by known methods to be infused. For example, soluble analogs can be obtained by treating the full length protein with standard detergents. Alternatively, a DNA construct can be prepared which contains the DNA encoding the functional protein, from which the membrane-spanning sequence of the gene is deleted, thereby rendering the expressed protein soluble and/or secretable through the endothelial cell membrane into the immediate environment within the vasculature.

The activity of *ecto*-ATP-diphosphohydrolases has been demonstrated on endothelial cells as well as leukocytes and platelets, and these proteins are believed to be widely distributed over the mammalian vascular endothelium. Partial internal amino acid sequence information following chymotryptic cleavage of an ATP diphosphohydrolase isolated from the particulate fraction of human term placenta is available [S. Christofiridis et al., Eur. J. Biochem. 134 (1) (November 15, 1995) 66-74]. Purification of bovine aortic and iliac endothelial ecto-ATPase was reported in a presentation

and abstract by J. Sévigny et al. (University of Sherbrooke, Canada) at the IBC Anticoagulant and Antithrombotic Meeting in Boston, October 24-25, 1994. Additionally, S.H. Lin and G. Guidotti, J. Biol. Chem. 264 (1989) 14408-14414 reported possession of rat liver CAM-105 cDNA and polyclonal antibodies, as well as identifying a consensus sequence (GPAYSGRET, amino acids 92-100) within the protein, and prepared oligonucleotide primers corresponding to nucleotides -40 to -24 (5') and 473 to 496 (3'); see also C.J. Sippel et al., J. Biol. Chem. 264 (1994) 2800-2826; Cheung et al., J. Biol. Chem. 268 (1993) 24303-24310. Further work has been reported in connection with the characterization of an ATP diphosphohydrolase active in rat blood platelets, S.S. Frasetto et al. Molec. Cell. Biochem. 129 (1993) 47-55; the characterization of ATP-diphosphohydrolase activities in the intima and media of the bovine aorta, Y.P. Côté et al., Biochimica et Biophysica Acta 1139 (1992) 133-142; the purification of ATP diphosphohydrolase from bovine aorta microsomes, K. Yagi et al., Eur. J. Biochem. 180 (1989) 509-513; and the characterization and purification of a calcium-sensitive ATP diphosphohydrolase from pig pancreas, LeBel et al., J. Biol. Chem. 255 (1980) 1227-1233.

Further available to the worker in the art are cDNA libraries of bovine and human liver endothelium (e.g. obtained and developed from Clontech, Palo Alto, CA, USA).

Isolation of porcine or human ecto-ATP diphosphohydrolase is carried out e.g. as described by Y.P. Côté et al., supra or J. Sévigny et al., supra, utilizing FSBA labelling and immunodetection. 5'-Fluorosulfonylbenzoyladenosine (FSBA) is a specific antagonist of ectoADPase. Specific activity of the enzyme is determined as described by LeBel et al., supra.

Following the protein purification, the protein sequence of, for example, the bovine species can be determined using standard, commercially available methodology, e.g. an Applied Biosystems Sequenator. Concurrently, polyclonal antibodies are raised against the bovine ATP diphosphohydrolase protein. Monoclonal and/or polyclonal antibodies are raised against the protein by techniques disclosed, for example, by Lin and

Guidotti, supra, and Cheung et al., supra. With monoclonal, and previously described polyclonal, antibodies in hand, together with a knowledge of at least a part of the protein sequence, there are two approaches to obtaining the gene in bovine, porcine or human cells:

(i) utilizing an expression library, the available antibodies are used to detect the colony including the cDNA encoding for the ATP diphosphohydrolase; and

(ii) utilizing defined oligomers corresponding to the amino acid sequences that have been obtained, to obtain the correct cDNA elements. See e.g. Lin and Guidotti, supra, and Cheung et al., supra.

The porcine cDNA sequence can be obtained by similar techniques as described above by probing with suitable antibodies or oligomers. Likewise the human ecto-ATP diphosphohydrolase protein can be determined following the procedures defined above, or alternatively by probing human cDNA from endothelial cells or genomic libraries.

Thereafter the entire length of cDNA can be sequenced by known methods (N. Rosenthal, NEJMed. 332 [March 2, 1995] 589-591). The obtained native cDNA can also be expressed recombinantly in E. coli.

The above procedures are well-described by Sambrook, Fritsch and Maniatis, Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA (1989).

The distribution of **CD39 protein** on B lymphocytes, activated NK cells, and certain T cell and endothelial cell lines (see Plesner, Inter. Rev. Cytology 158 (1995) 141-214; Maliszewski et al. supra; Kansas et al., J. Immunol. 146 (1991) 2235-44) is consistent with the known distribution of ecto-ADPases. The cell surface glycoprotein CD39 has two potential transmembrane regions, and binding by certain antibodies triggers signal transduction. The reported molecular mass of the native CD39 protein is 70-100 kD with 6 potential N-glycosylation sites and an observed molecular mass of 54kD after enzymatic removal of N-linked sugars

(Maliszewski et al., supra). Additionally, there are several potential targets for oxidative damage as the available deduced sequence data show that the protein is rich in cysteine (n=11), methionine (n=12) and tyrosine (n=27).

CD39 in a similar fashion to other markers is designated as a B cell activation marker (Engel et al., Leukemia & Lymphoma 1 [1994], 61-4). CD39 has been shown to have partial identity with yeast guanosine diphosphatases but no specific function has been yet assigned although a role in the mediation of homotypic B cell adhesion and antigen-specific responses has been described (Maliszewski et al., supra; Kansas et al., supra). The antigen has been found expressed on endothelial cells where activation related changes have been mentioned, in association with over 120 other potential markers (Favaloro, Immun. Cell Biol. 71 (1993) 571-581), and has been noted to be expressed on vascular endothelium, particularly in cutaneous vessels (Kansas et al., supra).

Once the native protein of interest is sequenced, it can be **derivatized** (i.e. mutated or truncated or otherwise altered by known procedures) for the purpose of increasing resistance to oxidative stress.

Examples of involved physiological oxidants against which oxidation-resistance is desirably maintained are superoxide and hydroxyl radicals and related species such as hydrogen peroxide and hypohalous acid. Oxygen free radical intermediates, such as superoxide and hydroxyl radicals, are produced through normal and pathologic metabolic processes.

Of the amino acids that make up proteins, histidine, methionine, cysteine, tryptophan and arginine are the most likely to be oxidized. For example, oxidation of methionines of a native protein may cause the protein to lose activity. Tyrosine is susceptible to nitric oxide and peroxynitrate, which could also thereby inactivate enzyme function. Therefore, in such cas different amino acids can be substituted for the native methionines, as described by e.g. C.B. Glaser et al., USP 5'256'770.

Methods for rendering amino acids resistant to oxidation are generally known. A preferred method is by removing the affected amino acid or replacing it with one or more different amino acids that will not react with oxidants. For example, the amino acids leucine, alanine and glutamine are preferred replacement amino acids based on size and neutral character. Methods by which amino acids can be removed or replaced in the sequence of a protein are also known to the skilled worker. Genes encoding a peptide with an altered amino acid sequence can be made synthetically [see e.g. Higuchi, PCR Protocols, Acad. Press., San Diego, USA (1990) 177-183]. A preferred method comprises site-directed in vitro mutagenesis, which involves the use of a synthetic oligodeoxy- ribonucleotide containing a desired nucleotide substitution, insertion or deletion designed to specifically alter the nucleotide sequence of a single-stranded target DNA. This primer, when hybridized to a single-stranded template with primer extension, results in a heteroduplex DNA which, when replicated in a transformed cell, encodes a protein sequence with the intended mutation.

A mutant ecto-ATPase analog that retains at least about 60%, and more preferably at least 70%, and even more desirably at least 90%, of normal activity after exposure to oxidants, can be considered to be substantially oxidation-resistant.

The invention also provides for **pharmaceutical compositions** having platelet aggregation inhibitory activity comprising a sterile preparation of a unit dose of a soluble, preferably oxidation-resistant, ecto-ATP diphosphohydrolase analog in a pharmaceutically acceptable carrier.

Administration of such analogs can be by a bolus intravenous injection, by a constant intravenous infusion, or by a combination of both routes.

The invention also contemplates **biocompatible materials**, such as prosthetic devices, which are coated with an oxidation resistant *ecto*-ATP diphosphohydrolas analog, see e.g. R.K. Ito et al., USP 5'126'140.

The present invention broadly includes a **method of tr ating the dysfuncti nal or activation response of a mammalian cell** (e.g. an endothelial cell) **to an inflammatory or other platelet-mediated activation stimulus**, comprising modifying such cell by inserting therein DNA encoding a polypeptide having ATP diphosphohydrolase activity, in operative association with a suitable promoter, and secreting and/or expressing functional ecto-ATPase from said cells at effective levels whereby platelet aggregation at the cell surface is inhibited.

The invention also includes the **cells so modified, and tissues or organs** comprising such cells.

Cells or cell populations can be treated in accordance with the present invention in vivo or in vitro (ex vivo). For example, for in vivo treatment, ecto-ATP diphosphohydrolase vectors can be inserted by direct infection of cells, tissues or organs in situ. Thus, the blood vessels of an organ (e.g., kidney) can be temporarily clamped off from the blood circulation of the patient, and the vessels perfused with a solution comprising a transmissible **vector construct** containing the ecto-ATP diphosphohydrolase gene, for a time sufficient for at least some of the cells of the organ to be genetically modified by insertion therein of the vector construct; and on removal of the clamps, blood flow can be restored to the organ and its normal functioning resumed.

Adenoviral mediated gene transfer into vessels or organs by means of transduction perfusion, as just described, is a means of genetically modifying cells in vivo.

The invention in a further aspect comprises a **method for inhibiting platelet aggregation or thrombus formation** in a subject in need of such therapy, which comprises inserting into cells of the suject which are under platelet-mediated activation or inflammation, DNA encoding a polypeptide having ATP diphosphohydrolase activity, in operativ association with a promoter, and expressing the polypeptide at platelet-aggregation (thrombus-suppressing) effective l vels.

In another aspect, cells can be removed from the subject or a donor animal, **genetically modified ex vivo** by insertion of vector DNA, and then re-implanted into the subject or transplanted into another recipient. Thus for example, an organ can be removed from a patient or donor, subjected ex vivo to the perfusion step previously described, and the organ can then be re-grafted into the patient or implanted into a different recipient of the same or different species.

Ex vivo genetically modified endothelial cells may be administered to a patient by intravenous or intra-arterial injection under defined conditions.

In still another embodiment, the invention comprises a **method for transplanting donor cells, or tissue or organs** comprising such cells, into a mammalian recipient in whom these cells are susceptible to a platelet-mediated activation stimulus, which comprises:

(a) modifying the donor cells, or progenitor cells thereof, by introducing therein DNA encoding a protein having ATP diphosphohydrolase activity; and

(b) transplanting the so-modified donor cells, tissue or organ into the recipient and expressing the polypeptide having ATP diphosphohydrolase activity, whereby recipient platelet aggregation at the surface of the cells is reduced or inhibited.

The donor species may be any suitable species which is the same or different from the recipient species and which is able to provide the appropriate endothelial cells, tissue or organs for transplantation or grafting.

In a preferred embodiment, human ecto-ATP diphosphohydrolase is expressed from cells of a different mammalian species, which cells have been placed or grafted into a human recipient. The donor may be of a species which is allogeneic or xenogeneic to that of the recipient. The recipient is a mammal, e.g. a primate, and is primarily human. However, other mammals, such as non-human primates, may be suitable recipients. For human recipients, it is envisaged that human (i.e. allogeneic) as well as pig (i.e. xenogeneic)

donors will be suitable, but any other mammalian species (e.g. bovine or non-human primate) may also be suitable as donor. For example, porcine aortic endothelial cells (PAEC), or the progenitor cells thereof, can be obtained from porcine subjects, genetically modified, and reimplanted into either the autologous donor (until a time suitable to be recruited for transplantation) or transplanted into another mammalian (i.e. human) subject.

The donor cells or tissue may be somatic recombinants or transgenic in the sense that they contain and express DNA encoding ecto-ATP diphosphohydrolase protein of a graft recipient of a different species in whom they are, or will be, implanted. Such cells or tissue may continue to express the desired ecto-ATP diphosphohydrolase indefinitely for the life of the cell. For example, porcine aortic endothelial cells (PAEC), or the progenitor cells thereof, can be genetically modified to express porcine or human ATP diphosphohydrolase protein at effective levels, for grafting into a human recipient.

Heterologous genes can be inserted into germ cells (e.g. ova) to produce **transgenic animals** bearing the gene, which is then passed on to offspring. For example, DNA encoding ATP diphosphohydrolase can be inserted into the animal or an ancestor of the animal at the single-cell or the early morula stage. The preferred stage is the single-cell stage although the process may be carried out between the two and eight cell stages. Methods of preparing transgenic pigs are discussed in W.L.Fodor and S.P.Squinto, Xeno 3 (1995) 23-26 and the references cited therein.

In another aspect genes can be inserted into somatic/body cells of the donor animal to provide a **somatic recombinant animal**, from whom the DNA construct is not capable of being passed on to offspring [see e.g. A.D. Miller and G.T. Rosman, Biotechniques 7, No. 9 (1989) 980-990].

Preferably, the inserted DNA sequences are incorporated into the genome of the cell. Alternatively, the inserted s quences may be maintained in the cell extrachromosomally,

either stably or for a limited period.

Cells, tissue or organs may be removed from a donor and grafted into a recipient by well-known surgical procedures. Although any mammalian cell can be targeted for insertion of the ecto-ATP diphosphohydrolase gene, endothelial cells are the preferred cells for manipulation. Modification of endothelial cells can be by any of various means known to the art. In vivo direct injection of cells or tissue with DNA can be carried out, for example. Appropriate methods of inserting foreign cells or DNA into animal tissue include microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection-k, transduction, retroviral infection, etc.

In another embodiment, the gene is inserted into a particular locus, e.g. the thrombomodulin locus, or locus containing von Willebrand factor. To prepare transgenic animals with such a gene, the construct is introduced into embryonic stem (ES) cells, and the resulting progeny express the construct in their vascular endothelium.

For gene delivery, **retroviral vectors**, and in particular, replication-defective retroviral vectors lacking one or more of the gag, pol, and env sequences required for retroviral replication, are well-known to the art and may be used to transform endothelial cells. PA317 or other producer cell lines producing helper-free viral vectors are well-described in the literature.

A representative retroviral construct comprises at least one viral long terminal repeat and promoter sequences upstream of the nucleotide sequence of the therapeutic substance and at least one viral long terminal repeat and polyadenylation signal downstream of the therapeutic sequence.

Vectors derived from adenoviruses, i.e. viruses causing upper respiratory tract disease and also present in latent infections in primates, are also generally known to the art and are useful in certain circumstances, particlarly in view of their ability to infect nonreplicating somatic cells. The ability of adenoviruses to attach to cells at low ambient

temperatures is also an advantage in the transplant setting which can facilitate gene transfer during cold preservation.

Prior to implantation, the treated endothelial cells or tissue may be screened for genetically modified cells containing and expressing the construct. For this purpose, the vector construct can also be provided with a second nucleotide sequence encoding an expression product that confers resistance to a selectable marker substance. Suitable selectable marks for screenng include the neo gene, conferring resistance to neomycin or the neomycin analog G418.

Alternative means of targeted gene delivery comprise DNA-protein conjugates, liposomes, etc.

The protein encoding region and/or the promoter region of the inserted DNA may be heterologous, i.e. non-native to the cell. Alternatively, one or both of the protein encoding region and the promoter region may be native to the cell, provided that the promoter is other than the promoter which normally controls ATP diphosphohydrolase expression in that cell.

The protein coding sequence may include sequence coding for an appropriate signal sequence, e.g. a nucleus-specific signal sequence.

Means to achieve thrombus-suppressing effective (i.e. "stable") levels of expression of an ATP hydrolyzing protein such as CD39 under endothelial activating conditions are also available.

Preferably the protein encoding region is under the control of a **constitutive or inducible** (i.e. a subset of "regulable") **promoter**.

An advantage of employing an inducible promoter for transplantation purposes is that the desired high level transcription/expression of the active gene/protein can be delay d for a suitabl period of time before grafting. For example, transcription can be obtained on demand in response to a predetermined stimulus, such as, e.g. the presence of

tetracycline in the cellular environment. An example of a tetracycline-inducible promoter which is suitable for use in the invention is disclosed by Furte et al., PNAS USA 91 (1994) 9302-9306. Alternatively, a regulable promoter system in which transcription is initiated by the withdrawal of tetracycline is described by Gossen and Bujard, PNAS USA 90 (1992) 5547-51.

Preferably, transcription/expression of the ATP diphosphohydrolase gene/protein is induced in response to a predetermined external stimulus, and the stimulus is applied beginning immediately prior to subjecting the cells to an activating stimulus, so that expression is already at effective levels for platelet aggregation-suppressing purposes. For example, cells of a donor mammal (e.g. porcine) may be genetically modified according to the invention by insertion of the ATP diphosphohydrolase gene (e.g. porcine or human) under the control of a promoter which is inducible by a drug such as e.g. tetracycline. The animal, whether somatic recombinant or transgenic, may be raised up to the desired level of maturity under tetracycline-free conditions until such time as the cells, or tissue or organs comprising the cells, are to be surgically removed for transplantation purposes. In such case, prior to surgical removal of the organ, the donor animal may be administered tetracycline in order to begin inducing high levels of transcription/ expression of the ATP hydrolyzing gene/protein. The organ can then be transplanted into a recipient (e.g. a human) and tetracycline may continue to be administered to the recipient for a sufficient time to maintain the ATP diphosphohydrolase protein at the desired levels in the transplanted cells to inhibit platelet aggregation in the recipient. Alternatively the organ, after being surgically removed from the donor, can be maintained ex vivo in a tetracycline-containing medium until such time as grafting into a recipient is appropriate.

In another embodiment transcription may be provided to occur as a result of withholding tetracycline from the cellular environment. Thus, cells of a donor animal may be

genetically modified according to the invention by insertion of a gene encoding an ATP diphosphohydrolase protein under the control of a promoter which is blocked by tetracycline, and which is induced in the absence of tetracycline. In such case the animal may be raised up to the desired level of maturity while being administered tetracycline, until such time as the cells, tissue or organs are to be harvested. Prior to surgical removal, the donor animal may be deprived tetracycline in order to begin inducing expression of ATP diphosphohydrolase protein, and the patient in whom the cells, tissue or organs are transplanted may thereafter also be maintained tetracycline-free for a sufficient time to maintain appropriate ATP diphosphohydrolase levels of expression.

In addition to using a constitutive or inducible promoter facilitating high level expression, multiple copies of DNA encoding ATP diphosphohydrolase may be placed in operative association with such a promoter to further increase gene transcription and protein expression.

It will be appreciated that in xenotransplantation the modified cells and donor tissue and organs defined above have a supplementary function in the prevention of transplant rejection since the primary response is hyperacute rejection. Therefore, the genetic material of the cells of the donor organ is typically also altered such that activation of the complement pathway in the recipient is prevented. This may be done by providing transgenic animals that express the complement inhibitory factors of the recipient species. The endothelial cells of a donor organ obtained from such an animal can be modified by gene therapy techniques to provide the endothelial cells defined above. Alternatively a vector containing DNA encoding a protein having ATP diphosphohydrolase activity can be introduced into the transgenic animal at the single cell or the early morula stage. In this way the resulting transgenic animal will express the complement inhibitory factors and will have endothelial cells as defined above. Thus in a further aspect the invention also provides **endothelial cells, tissue, donor**

organs and non-human transgenic or somatic recombinant animals as defined above which express one or more human complement inhibitory factors.

Although any mammalian cell can be targeted for insertion of the ATP diphosphohydrolase gene, such as monocytes, NK cells, lymphocytes, or islet cells, the preferred cells for manipulation are endothelial cells.

In an alternative embodiment of the invention, the polypeptide having ATP diphosphohydrolase activity, in a pharmaceutically acceptable carrier, may be applied directly to cells, tissue or organs in vivo.

Thus the invention also comprises a **method of inhibiting platelet aggregation** in a warm-blooded mammal comprising administering to that mammal an effective amount for inhibiting platelet aggregation of a polypeptide having ATP diphospho- hydrolase activity (e.g. CD39), or a pharmaceutically acceptable salt thereof, in a pharmaceutically acceptable carrier.

The invention additionally comprises a **pharmaceutical composition** having anti-platelet aggregatory activity comprising a unit dose of a polypeptide having ATP diphosphohydrolase activity (e.g. CD39), or pharmaceutically acceptable salt thereof, in a pharmaceutically acceptable carrier.

A polypeptide according to the invention or a hydrohalic acidic derivative thereof is typically administered as a pharmaceutical composition in the form of a solution or suspension. However, as is well known, peptides can also be formulated for therapeutic administration as tablets, pills, capsules, sustained release formulations or powders. The preparation of therapeutic compositions which comprise polypeptides as active ingredients is well understood in the art. Typically, such compositions are prepared in injectable form, e.g. as liquid solutions or suspensions.

A pharmaceutical composition useful in the practice of the present invention can contain a polypeptide having ATP diphosphohydrolase activity formulated into a therapeutic composition as a neutralized pharmaceutically acceptable salt form. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the polypeptide), and which are formed with inorganic acids such as hydrochloric or phosphoric acid, or organic acids such as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases, such as sodium, potassium, ammonium, calcium or ferric hydroxides, or organic bases such as isopropylamine, trimethylamine, (2-ethylamino)ethanol, histidine or procaine.

The therapeutic peptide-containing composition is conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" refers to physically discrete units suitable as unitary dosages for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required excipient.

The compositions are administered in a manner compatible with the dosage formulation and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, the capacity of the subject's blood hemostatic system to utilize the active ingredient, and the degree of platelet aggregation inhibition desired. The precise amount of active ingredient required to be administered depends on the judgment of the practitioner and is peculiar to each individual. However, suitable dosage ranges are of the order of one to hundreds of nanomoles of polypeptide per kilogram body weight per minute, and depend on the route of administration.

Also within the scope of the invention is a **vascular prothesis** having applied thereto a polypeptide having ATP diphosphohydrolase activity (e.g. CD39). Commercially available materials suitabl for preparing such a prosthesis include a polyester such as Dacron® (C.R. Bard) or a

polyfluorocarbon such as Teflon (Gore-Tex).

The present inv ntion may be applied in the therapeutic treatment of a wide variety of disease states in mammals where there is an increase in propensity for platelet aggregation, (e.g. atherosclerotic and thrombotic conditions, such as ischemic heart disease, atherosclerosis, multiple sclerosis, intracranial tumors, thromboembolism and hyperlipemia, thrombophlebitis, phlebothrombosis, cerebral thrombosis, coronary thrombosis and retinal thrombosis), as well as following parturition or surgical operations such as coronary artery bypass surgery, angioplasty, or prosthetic heart valve implantation.

The following Examples are illustrative only and not limitative of the invention.

Example 1(a):

Xenogeneic quiescent porcine aortic endothelial cells (PAEC) in the absence of plasma xenoreactive antibodies and complement exert an inhibitory effect on human platelet activation responses to standard platelet agonists.

The factor inhibitory to human platelet activation in in vitro systems is cell-associated and not found in cell culture supernatants. This cell-associated factor completely blocks human platelet responses to ADP (2-10 µM), collagen (2-10 µg/ml) and low concentrations of thrombin (<1 U/ml) in the presence of PAEC in monolayer, on bead cultures or cell suspensions.

The importance of prostacycline metabolites, thrombomodulin (by thrombin neutralization) and NO have been evaluated by several methodologies and shown not to be crucial for this inhibition of platelet activation processed by PAEC.

In view of the demonstrable non-inhibitable effects of ADP-β-S (a non-hydrolyzable analogue of ADP which is thus not degraded by the ecto-ADPases) on human platelet responses in association with PAEC in the experimental systems examined, the inhibitory endothelial cell associated factor is identified as an ecto-ATP diphosphohydrolase (apyrase).

Example 1(b): Loss of inhibitor phenotype of PAEC following PAEC activation

Activation of PAEC by standardized human recombinant in vitro results in rapid loss, within 30 to 60 minutes, of the EC antiaggregatory phenotype with the development of a permissive environment for platelet activation.

Example 1(c): Modulation of ecto-ATP diphosphohydrolases on PAEC by rTNFa

The endothelial cell ecto-ATP diphosphohydrolase is significantly modulated by EC activation responses.

Kinetics of ecto-ATP diphosphohydrolase: as determined by catabolism of ^{14}C-ADP, PAEC ecto-ATP diphosphohydrolase Vmax is of the order of 50-55 nmol ADP converted per 1 x 10^6 cells/min (Km approximately 200 µM). These figures are in concordance with those stated for human umbilical vein EC and previously for porcine EC as determined by other methodology [A.J. Marcus et al., J. Clin. Invest. 88 (1991) 1690-1696; E.L. Gordon et al., J. Biol. Chem. 261 (1986) 15496-15507].

Endothelial cells when activated by TNFα at 10 and 50 ng/ml lose ecto-ADPase activity after 60 minutes incubation. **FIG. 1** shows levels of enzyme activity at 4 hours as determined by biochemical methodology (D. LeBel et al., supra as well as TLC determination of cellular degradation of ^{14}C-ADP to AMP (A.J. Marcus et al., supra). Once EC are activated, there is loss of this inhibitory potential, and therefore platelet activation can occur. This inhibitory activity is chiefly related to ecto-ATP diphosphohydrolase expressed on PAEC.

Example 1(d):

PAEC ecto-ATP diphosphohydrolase kinetics after activation of intact cells was also determined by TLC: Vmax 15 nmol ADP / 1 x 10^6 cells/min (Km 70µM). Reciprocal plots suggest an uncompetitive inhibition process. This novel observation is in keeping with either an inhibitor binding to the enzyme-substrate complex (but not the free enzyme itself) or a process of inhibition which disturbs the enzyme catalytic function independently of substrate binding (**FIG. 2**).

Example 2(a): Oxidative stress inhibits porcine endothelial cell ecto-ATP diphosphohydrolase

Incubation of PAEC with HOOH (hydrogen peroxide) at concentrations of 5 µM and 10 µM which are potentially produced by activated endothelial cells, in the absence of catalase activity, has a significant effect on the activity of the ecto-ATP diphosphohydrolase comparable and non-additive to that observed following cell activation with cytokines. **FIG. 3** depicts loss of enzyme activity after treatment with 5 µM HOOH

after 4 hours incubation.

The generation of HOOH by PAEC following activation with cytokines such as TNF in vitro was determined to be of the order of about 0.015 nmoles/min/10^6 cells.

Ecto-ATP diphosphohydrolases could thus be sensitive to oxidation processes which are promoted by cytokine activation of PAEC. Endogenous xanthine oxidase and other, e.g. NADPH oxidase, enzyme systems in PAEC elaborate significant levels of reactive oxygen intermediates following cellular activation and these could have profound effects on membrane associated ectoenzymes.

Example 2(b):

In a reciprocal fashion to agents which induce oxidative stress, β-mercaptoethanol, a potent reducing agent in micromolar concentrations, protects the enzyme activity. This also holds for situations under which endothelial cells are activated by cytokines **(FIG. 4)**.

Example 2(c):

A loss of ecto-ATP diphosphohydrolase activity on PAEC is demonstrated as a result of TNFα activation and following incubation with and perturbation of endothelial cells by HOOH (hydrogen peroxide, 5 µM) and by xanthine oxidase/xanthine (XO/X, at combinations of 200 µM xanthine and typically 100 mU/ml of xanthine oxidase which is phosphate free) in vitro. XO/X cause oxidative damage to cells and their membrane proteins and lipids by both peroxide and superoxide radicals. In the presence of iron, toxic hydroxyl radicals are formed. Note the late decrease in enzyme activity following exposure to oxygen radicals **(FIG. 5)**.

Example 3:

Antioxidant strategies with SOD/catalase supplementation in the systems tested likewise are shown to be protective in preserving endothelial cell ecto-ATP diphosphohydrolase activity following activation processes. Superoxide dismutas (Cu-Zn form from bovine red blood cells) removes oxygen

radicals, and was used at a concentration of 330 U/ml. Catalase degrades HOOH, and a preparation from bovine liver was used at a final concentration of 1000 U/ml.

Zinc has diverse effects on cell membranes but can also serve as a potent antioxidant as potentially demonstrated here at concentrations previously documented to maintain porcine endothelial integrity following cytokine perturbation in vitro. Supplementation in these systems likewise appears to be protective in preserving endothelial cell ecto-ATP diphosphohydrolase activity (**FIG. 6**).

Example 4:

Direct oxidation of the endothelial cell ecto-ATP diphosphohydrolase is responsible for the modulation of endothelial cell - platelet interactions in the setting of cellular activation.

Experiments similar to those described above on the purified protein are performed to evaluate further the direct loss of activity following oxidation with or without further proteolytic modification [Rivett, Curr. Top. Cell. Regul. 28 (1986) 291].

Example 5:

FIG. 7 demonstrates loss of activity after 60 minutes warm ischaemic time and then in addition 5, 15, 30 and 60 minutes warm reperfusion in vivo. Note the loss in activity after 30 minutes reperfusion in vivo. Initial increases in ATP diphosphohydrolase activity could represent associated leucocyte adherence to injured endothelium in vivo.

Example 6:

FIG. 8 demonstrates that pretreatment of rats with cobra venom factor (CVF) to deplete animals of complement also results in systemic complement activation injury with induction of oxidative stress and as a consequence potentiates the loss of ATP diphosphohydrolase activity when glomeruli are rendered ischaemic and then reperfused for 30 minutes.

Example 7: Northern Analysis of CD39 in HUVEC following cytokine activation

Human umbilical vein endothelial cells (HUVEC) were incubated with TNFα (final concentration 10 ng/ml) for 2, 6 and 24 hours. Cells were washed twice with a phosphate buffer, RNA was purified and analysed by Northern blot. 10 µg of total RNA per well was applied on the TAE-agarose gel (TAE = tris/acetic acid/EDTA buffer). Electrophoresis was run at 40 mA for 2 hours. RNA was transferred to a charge-modified nylon membrane and UV-cross- linked. CD39 cDNA fragment cleaved from the plasmid DNA (pCDNA3-CD39) was labeled with [α^{32}P]-dCTP to a specific activity of 2 x 10^9 cpm/µg DNA, by the random hexamer labeling method. Prehybridization, hybridization, washes, and stripping of the membrane were carried out with the rapid hybridization protocol from Stratagene. Final washes were at 60°C in 0.1-x sodium saline citrate (SSC)/0.1% sodium dodecylsulfate (SDS). The blot was exposed to Kodak XAR-2 film with an intensifying screen at -80°C for 1 day. Results as depicted in **FIG. 9** show markedly decreased levels of CD39/ecto-ADPase mRNA following TNFα stimulation of EC at 6 hours and beyond to 24 hours.

Example 8: COS-7 cells transfected with CD39 have biochemical and functional activity of ecto-ADPase

COS-7 cells transfected with CD39 cDNA express immunologically identified CD39 as determined by FACS analysis (**FIG.10**).

Whole cell lysates (**FIG.11**) and membrane preparations (**FIG.12**) of COS-7 cells show significant activity only when COS-7 cells were transfected with CD39 vector as compared to empty vector or to control COS-7 cells. The estimation of ecto-ADPase activity was determined by hydrolysis of 200 µM ADP under Ca^{++}-dependent conditions.

Membrane preparations of COS-7 cells transfected with CD39 cDNA successfully abrogated platelet aggregation to ADP (5 µM) in vitro (**FIG.13**).

Sequence listing

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: Sandoz Ltd.
(B) STREET: Lichtstrasse 35
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(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): CH-4002
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(A) NAME: New England Deaconess Hospital Corporation
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(C) CITY: Boston
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(E) COUNTRY: U.S.A.
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(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 01966

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(B) STREET: 45, Longwood Avenue, Apt. 705
(C) CITY: Brookline
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 02146

(ii) TITLE OF INVENTION: GENE THERAPY FOR TRANSPLANTATION AND INFLAMMATORY OR THROMBOTIC CONDITIONS

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE:

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO PCT/EP 96/.....

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/410371
(B) FILING DATE: 24-MAR-1995
(A) APPLICATION NUMBER: US ...
(B) FILING DATE: 12-FEB-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1818 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE: human MP-1 B lymphoblastoid cell line
(A) ORGANISM: Homo sapiens

(x) PUBLICATION INFORMATION:
C.R. Maliszewski et al., J. Immunol. 153 (8) (1994) 3574-3583
(Fig. 2 on page 3577)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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   1  accacaccaa  gcagcggctg  gggggggaa  agacgaggaa  agaggaggaa  aacaaaagct
  61  gctacttatg  gaagatacaa  aggagtctaa  cgtgaagaca  ttttgctcca  agaatatcct
 121  agccatcctt  ggcttctcct  ctatcatagc  tgtgatagct  ttgcttgctg  tggggttgac
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 241  tcacacaagt  ttatacatct  ataagtggcc  agcagaaaag  gagaatgaca  caggcgtggt
 301  gcatcaagta  gaagaatgca  gggttaaagg  tcctggaatc  tcaaaatttg  ttcagaaagt
 361  aaatgaaata  ggcatttacc  tgactgattg  catggaaaga  gctagggaag  tgattccaag
 421  gtcccagcac  caagagacac  ccgtttacct  gggagccacg  gcaggcatgc  ggttgctcag
 481  gatggaaagt  gaagagttgg  cagacagggt  tctggatgtg  gtggagagga  gcctcagcaa
 541  ctacccctttt  gacttccagg  gtgccaggat  cattactggc  caagaggaag  gtgcctatgg
 601  ctggattact  atcaactatc  tgctgggcaa  attcagtcag  aaaacaaggt  ggttcagcat
 661  agtcccatat  gaaaccaata  atcaggaaac  ctttggagct  ttggaccttg  ggggagcctc
 721  tacacaagtc  acttttgtac  cccaaaacca  gactatcgag  tccccagata  atgctctgca
 781  atttcgcctc  tatggcaagg  actacaatgt  ctacacacat  agcttcttgt  gctatgggaa
 841  ggatcaggca  ctctggcaga  aactggccaa  ggacattcag  gttgcaagta  atgaaattct
 901  cagggaccca  tgctttcatc  ctggatataa  gaaggtagtg  aacgtaagtg  acctttacaa
 961  gacccccctgc  accaagagat  ttgagatgac  tcttccattc  cagcagtttg  aaatccaggg
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1381  tttcattggc  aagatccagg  gcagcgacgc  cggctggact  ttgggctaca  tgctgaacct
1441  gaccaacatg  atcccagctg  agcaaccatt  gtccacacct  ctctcccact  ccacctatgt
1501  cttcctcatg  gttctattct  ccctggtcct  tttcacagtg  gccatcatag  gcttgcttat
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1621  tggctggagt  gaggaaaaaa  tcgtccaggg  agcattttcc  tccatcgcag  tgttcaaggc
1681  catccttccc  tgtctgccag  ggccagtctt  gacgagtgtg  aagcttcctt  ggcttttact
1741  gaagcctttc  ttttggaggt  attcaatatc  ctttgcctca  aggacttcgg  cagatactgt
1801  ctctttcatg  agtttttc
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Translation (nucleotide positions 68 to 1597):

MEDTKESNVKTFCSKNILAILGFSSIIAVIALLAVGLTQNKALP
ENVKYGIVLDAGSSHTSLYIYKWPAEKENDTGVVHQVEECRVKGPGISKFVQKVNEIG
IYLTDCMERAREVIPRSQHQETPVYLGATAGMRLLRMESEELADRVLDVVERSLSNYP
FDFQGARIITGQEEGAYGWITINYLLGKFSQKTRWFSIVPYETNNQETFGALDLGGAS
TQVTFVPQNQTIESPDNALQFRLYGKDYNVYTHSFLCYGKDQALWQKLAKDIQVASNE
ILRDPCFHPGYKKVVNVSDLYKTPCTKRFEMTLPFQQFEIQGIGNYQQCHQSILELFN
TSYCPYSQCAFNGIFLPPLQGDFGAFSAFYFVMKFLNLTSEKVSQEKVTEMMKKFCAQ
PWEEIKTSYAGVKEKYLSEYCFSGTYILSLLLQGYHFTADSWEHIHFIGKIQGSDAGW
TLGYMLNLTNMIPAEQPLSTPLSHSTYVFLMVLFSLVLFTVAIIGLLIFHKPSYFWKD
MV

Claims

1. A **non-human transgenic or somatic recombinant mammal** comprising in its cells heterologous DNA encoding a polypeptide having activity of an ATP-diphosphohydrolase under cellular activating conditions.

2. A mammal of claim 1 in which the heterologous DNA is contained in its endothelial cells.

3. A mammal of claim 1 in which the polypeptide comprises human CD39 protein.

4. A mammal of claim 3 which is porcine.

5. A mammal of claim 4 in which the polypeptide comprises an oxidation-resistant analog of human CD39 protein.

6. **Cells, or tissue or organs comprising cells**, of a donor mammalian species, the cells, tissue or organs being modified to be capable of expressing DNA encoding a polypeptide having ATP-diphosphohydrolase activity at platelet-suppressing effective levels in a graft recipient of the same or a different species as the donor under cellular activating conditions.

7. Cells of claim 6, or tissue or organs comprising cells of claim 6, which are endothelial cells.

8. Cells, tissue or organs of claim 7 which are human.

9. Cells, tissue or organs of claim 7 which are porcine.

10. Endothelial cells, or tissue or organs comprising cells, capable of expressing heterologous DNA encoding a polypeptide having activity of an ATP-diphosphohydrolase under cellular activating conditions.

11. A **vector construct** for genetically modifying a mammalian cell to render it less susceptible to an inflammatory or immunological stimulus and platelet aggregation, which comprises DNA encoding a polypeptide having ATP diphospho-hydrolase activity, under the control of a promoter capable of initiating transcription of the DNA under conditions of cell activation or oxidative stress.

12. A vector construct according to claim 11 wherein the encoded polypeptide comprises human CD39 protein.

13. A vector construct according to claim 11 wherein the encoded polypeptide is under the control of an inducible promoter.

14. A **pharmaceutical composition** having platelet aggregation-inhibiting activity comprising a recombinant polypeptide having ATP diphosphohydrolase activity or pharmaceutically acceptable salt thereof, or an oxidation-resistant analog thereof, in a pharmaceutically acceptable carrier.

15. The pharmaceutical composition of claim 14 wherein the polypeptide comprises human CD39 protein.

16. A **prosthetic intravascular device** comprising a synthetic biocompatible material having applied thereto recombinant ATP diphosphohydrolase or an oxidation-resistant analog thereof.

17. A **method of genetically modifying a mammalian cell** to render it less susceptible to an inflammatory or immunological stimulus and platelet adhesion, which comprises conferring on such cell the capability of stably expressing a polypeptide having activity of an ATP diphosphohydrolase under cellular activating conditions.

18. A **method of genetically modifying a mammalian cell** to render it capable of inhibiting platelet aggregation, which comprises: inserting into the cell, or a progenitor thereof, DNA encoding a polypeptide having activity of an ATP diphosphohydrolase, and expressing the polypeptide from the cell under cellular activating conditions at platelet aggregation-suppressing effective levels.

19. The method of claim 17 or 18 wherein the polypeptide comprises human CD39 protein.

20. The method of claim 17 or 18 wherein the polypeptide is substantially oxidation-resistant.

21. The method of claim 17 or 18 wherein the polypeptide is in operative association with an inducible promoter.

22. A **method of controlling platelet aggregation and thereby preventing or alleviating a thrombotic condition in a mammalian subject in need of such therapy** which comprises: genetically modifying cells of the subject susceptible to platelet-mediated activation by inserting therein DNA encoding a polypeptide having ATP diphosphohydrolase activity, and expressing the polypeptide from the cells at platelet aggregation-suppressing effective levels.

23. The method of claim 22 in which the cells are endothelial cells.

24. The method of claim 22 in which the polypeptide comprises human CD39 protein.

25. The method of claim 22 wherein the subject is human.

26. The method of claim 22 in which the polypeptide is substantially oxidation-resistant.

27. A **method of transplanting donor allogenic or xenogen ic cells, or graftable tissue or organs comprising such cells**, to a mammalian recipient in whose blood or plasma these cells or tissue or organs are susceptible to an activation stimulus, which comprises:

(a) genetically modifying such donor cells, or progenitor cells thereof, by inserting therein DNA encoding a polypeptide having activity of an ATP diphosphohydrolase or an oxidation-resistant analog thereof in operative association with a promoter; and

(b) transplanting the resultant modified donor cells, tissue or organs into the recipient and expressing from the resultant modified cells or tissue or organs the polypeptide having ATP diphosphohydrolase activity at platelet-aggregation suppressing effective levels.

28. The method of claim 27 in which the cells are endothelial cells.

29. The method of claim 27 in which the polypeptide comprises human CD39 protein.

30. The method of claim 29 in which the recipient is human.

31. The method of claim 27 in which the polypeptide is substantially oxidation-resistant.

32. The method of claim 30 in which the donor is xenogenic as to the recipient.

33. The method of claim 30 in which the donor cells, tissue or organs are porcine.

34. A **method of inhibiting platel t aggregation and thereby treating thrombotic disorders in a mammalian subject**, comprising administering to the subject an amount effective for inhibiting platelet aggregation of a recombinant polypeptide having ATP diphosphohydrolase activity or pharmaceutically acceptable salt thereof, in a pharmaceutically acceptable carrier.

35. The method of claim 34 wherein the polypeptide comprises human CD39 protein.

36. The method of claim 34 wherein the subject is human.

37. **Use of a recombinant polypeptide having ATP diphosphohydrolase activity** or pharmaceutically acceptable salt thereof, or an oxidation-resistant analog thereof, in the preparation of a medicament for reducing platelet aggregation.

38. Use according to claim 37 wherein the polypeptide comprises human CD39 protein.

39. A **peptide analog of human CD39 protein** having activity of a native ATP-diphosphohydrolase and which is substantially oxidation-resistant.

40. A peptide analog according to claim 39 which is soluble.

41. A peptide analog according to claim 40 which is essentially free of membrane-spanning domains.

FIG. 1

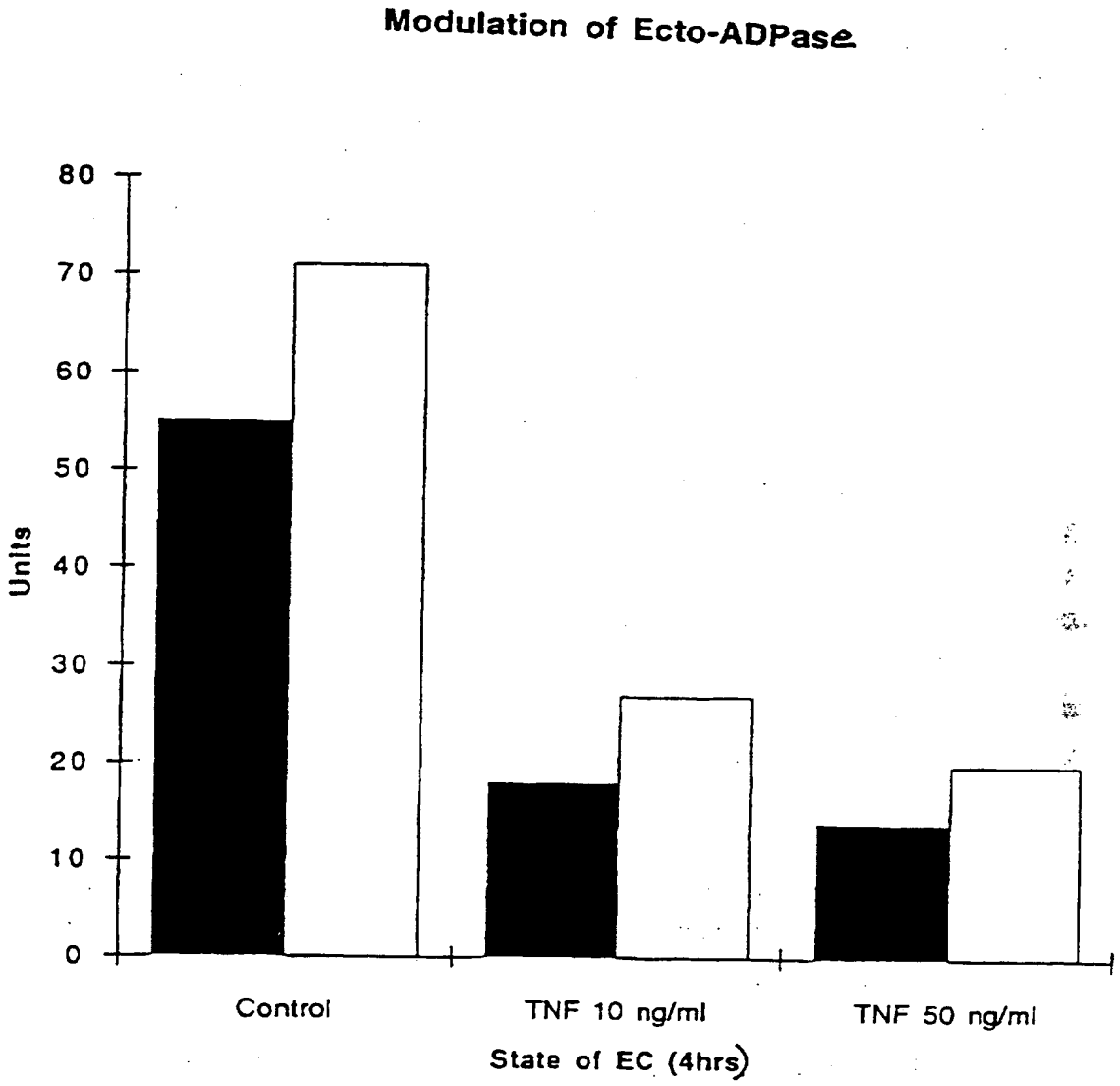


FIG. 2

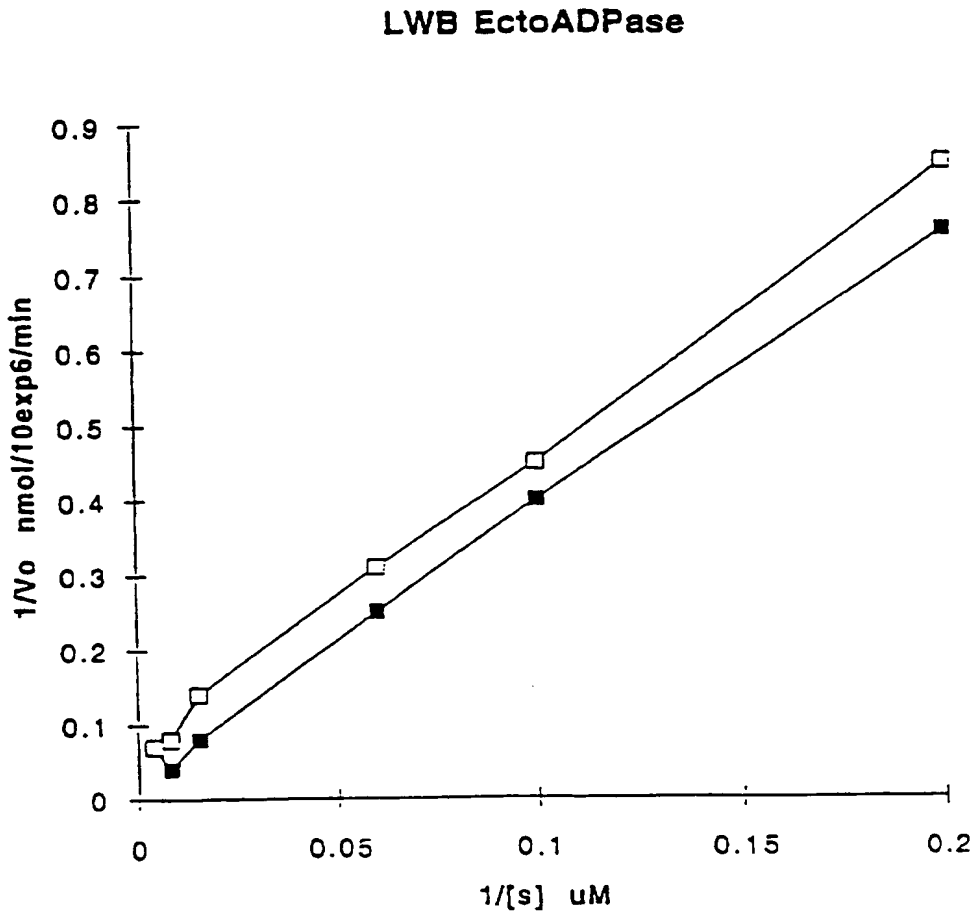


FIG. 3

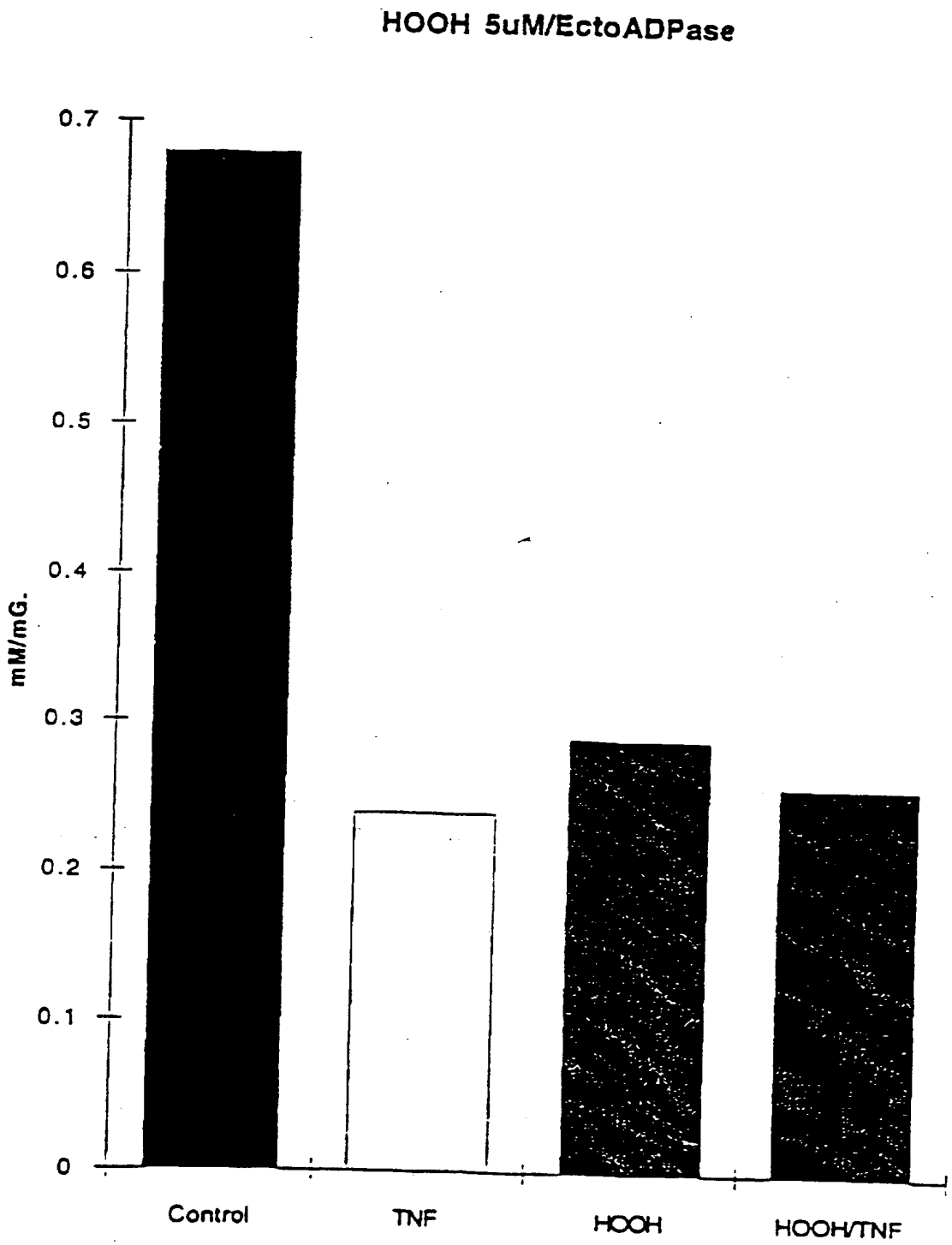


FIG. 4

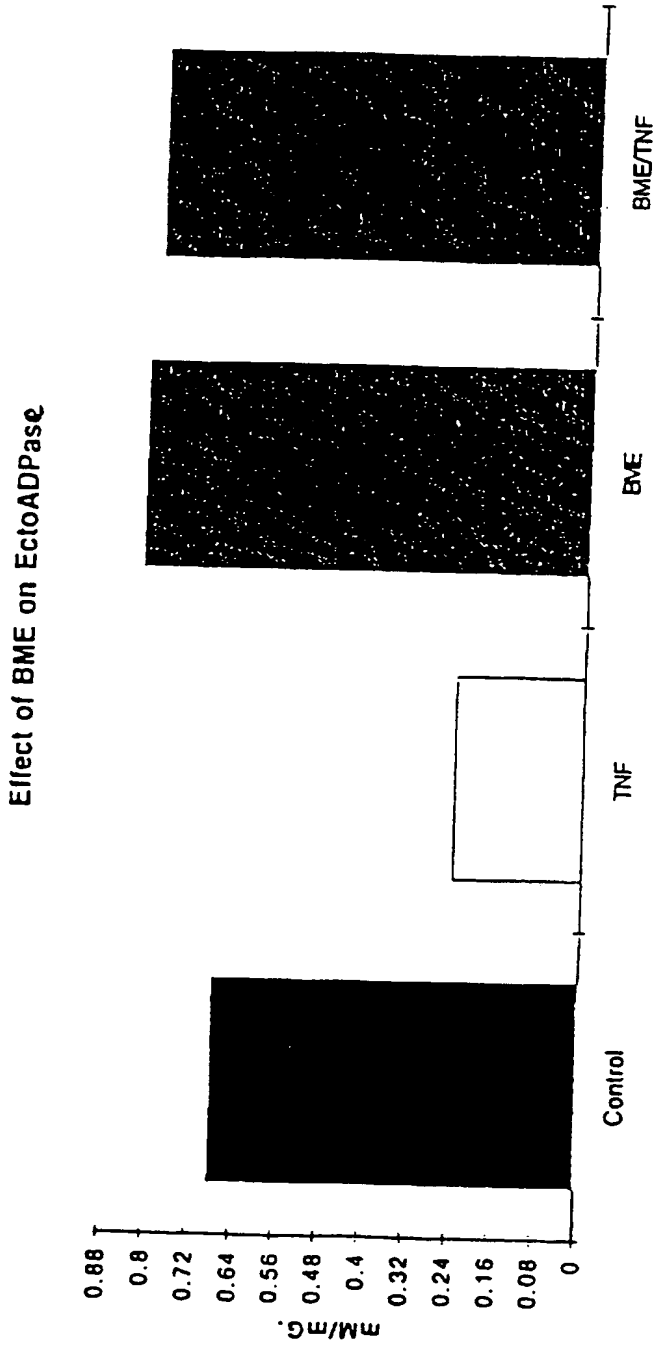
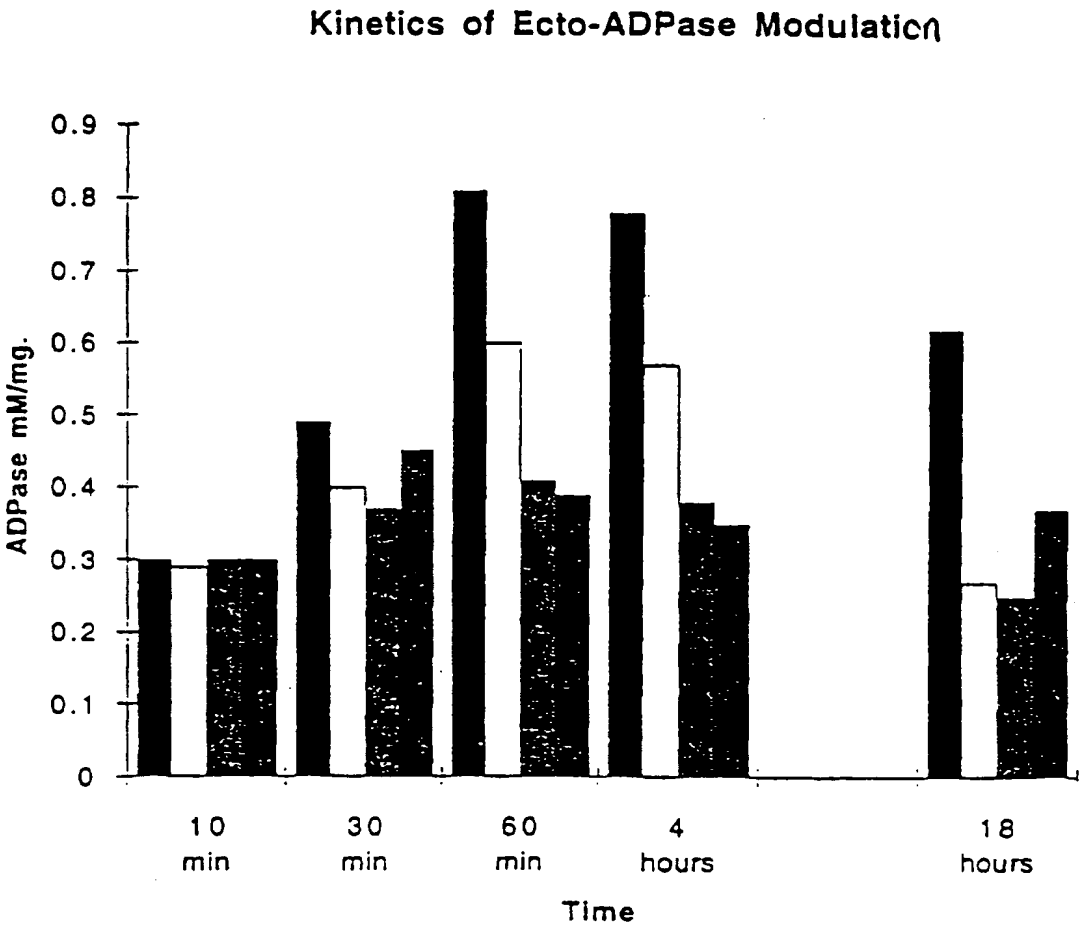


FIG. 5



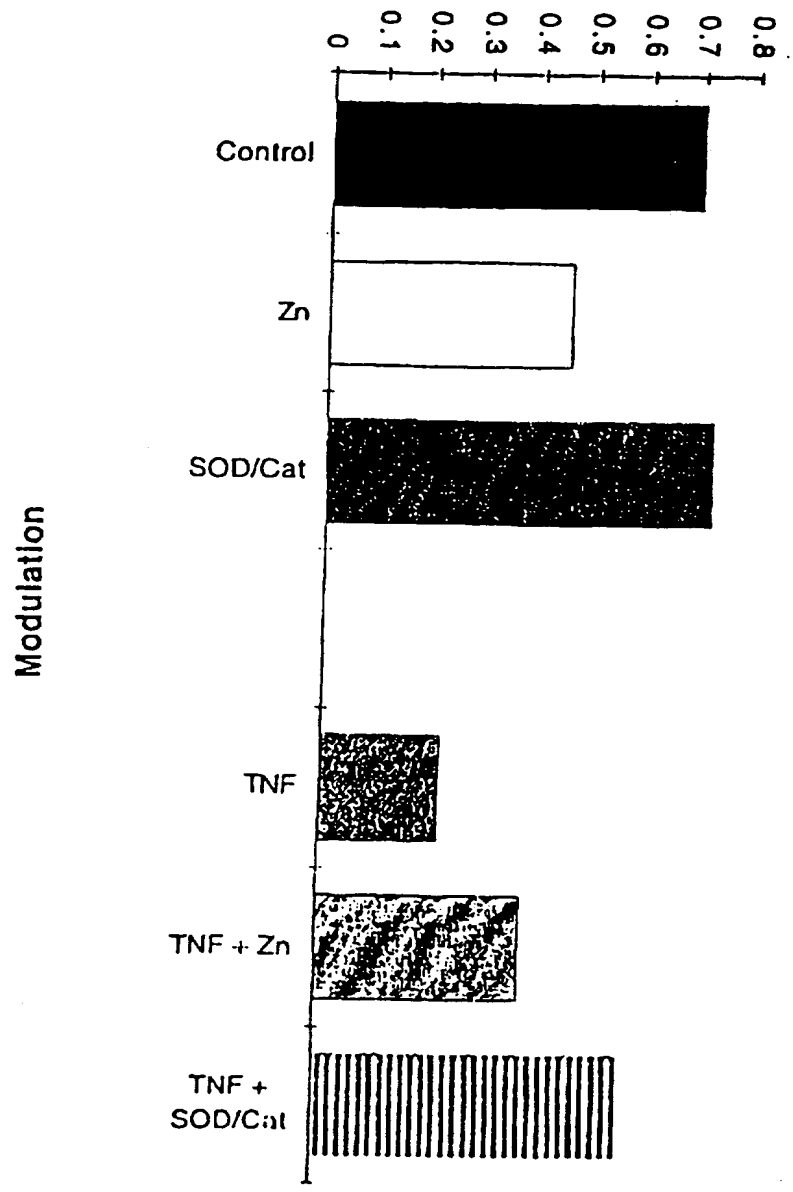


FIG. 6

FIG. 7

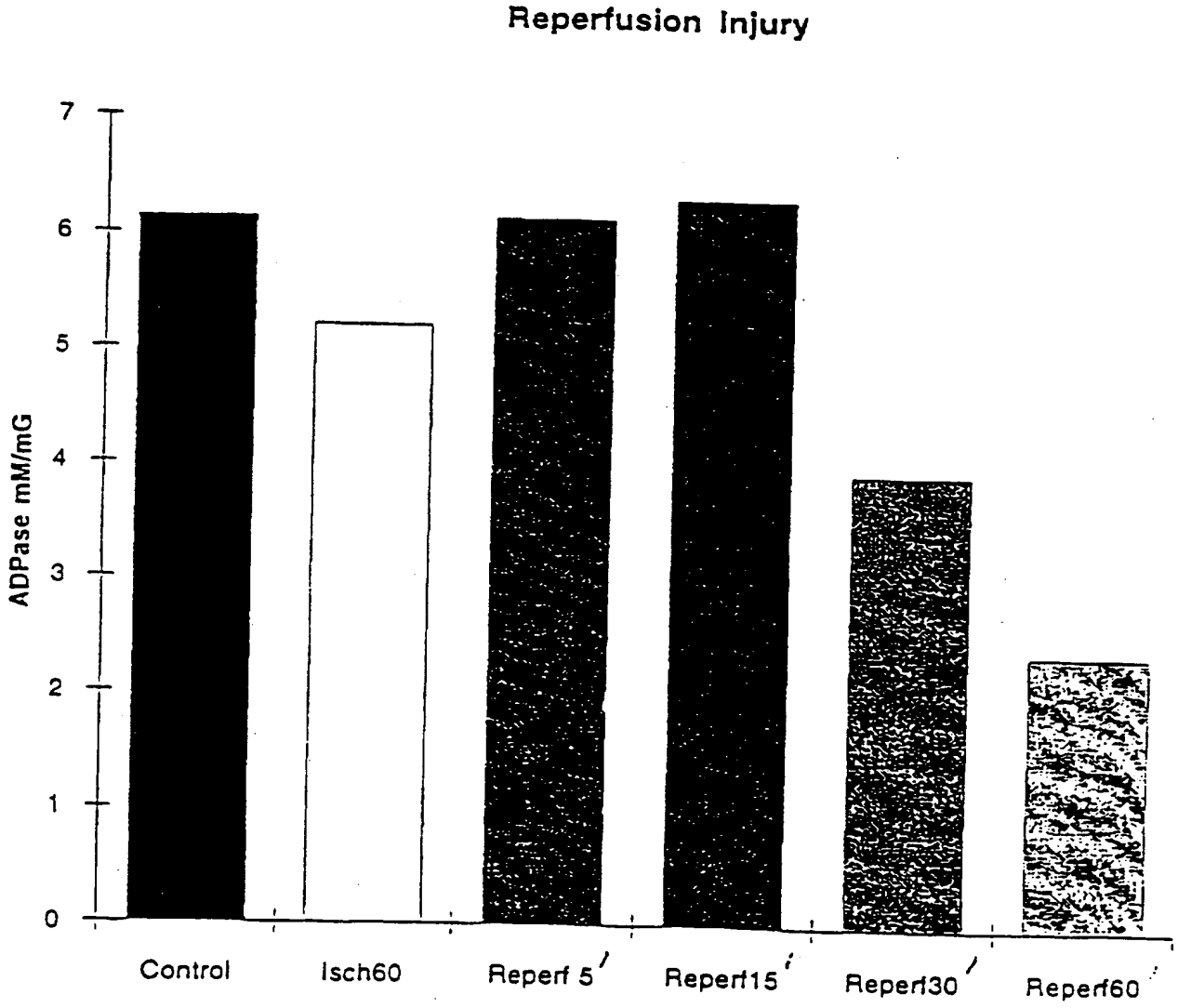


FIG. 8

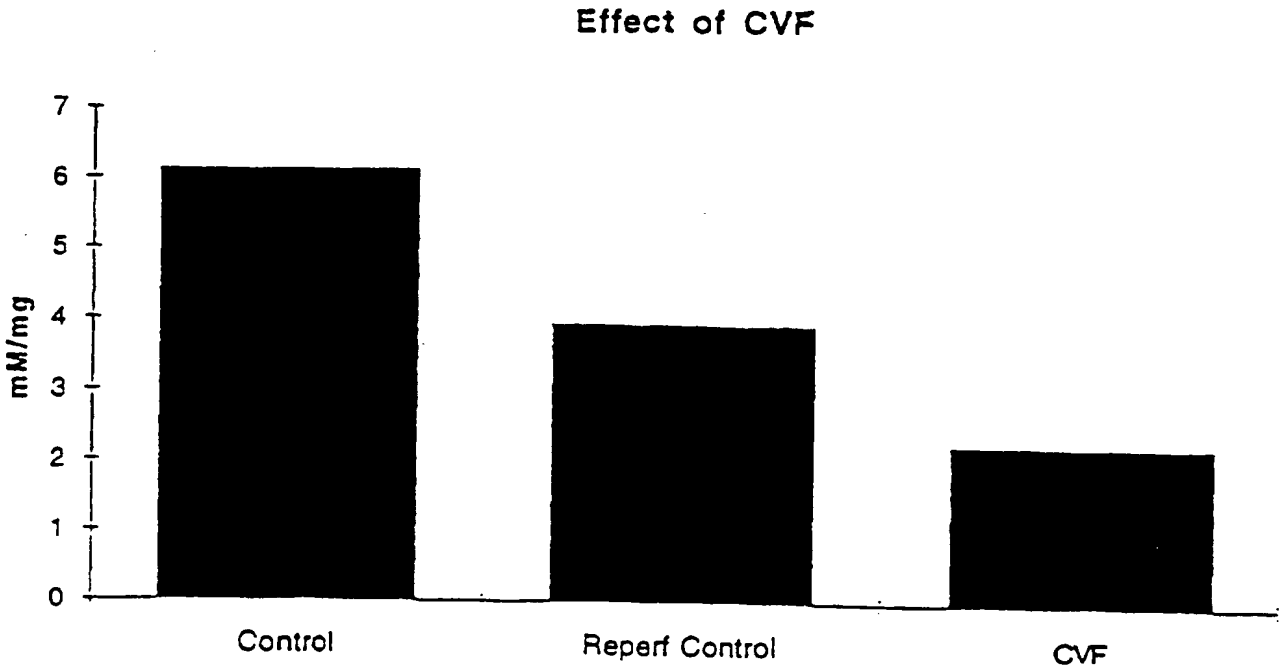


FIG. 9

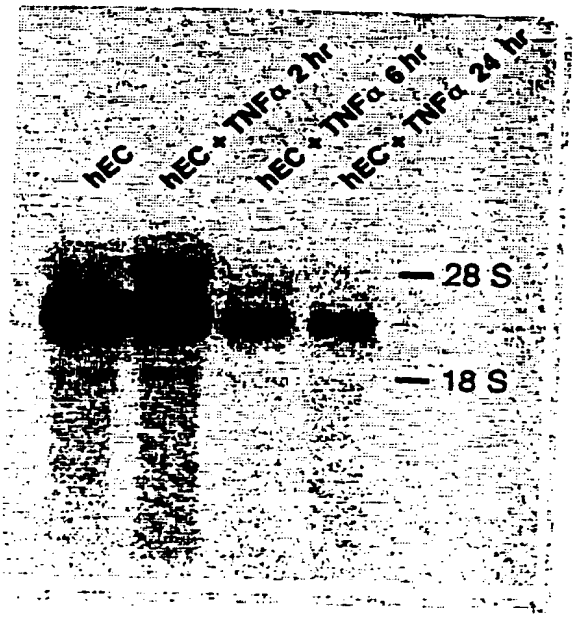


FIG. 10

Transient tranfection of COS-7 cells with pCDNA3/CD39

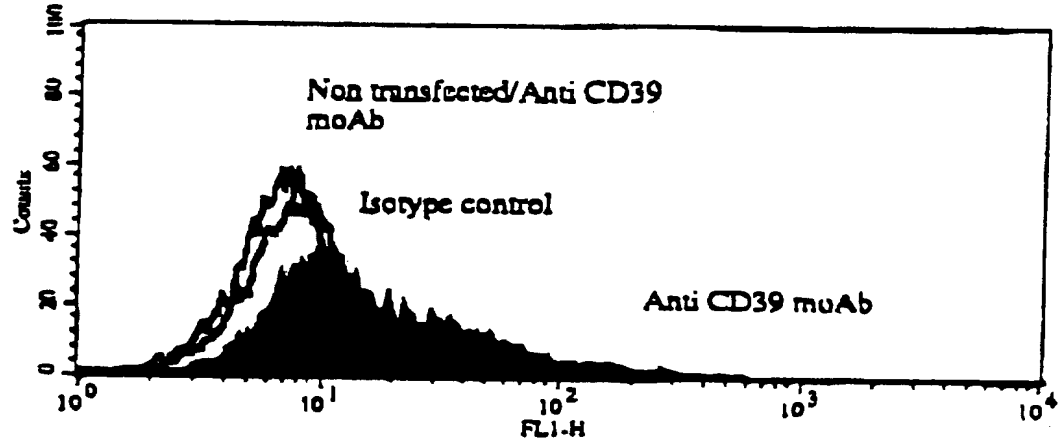


FIG. 11

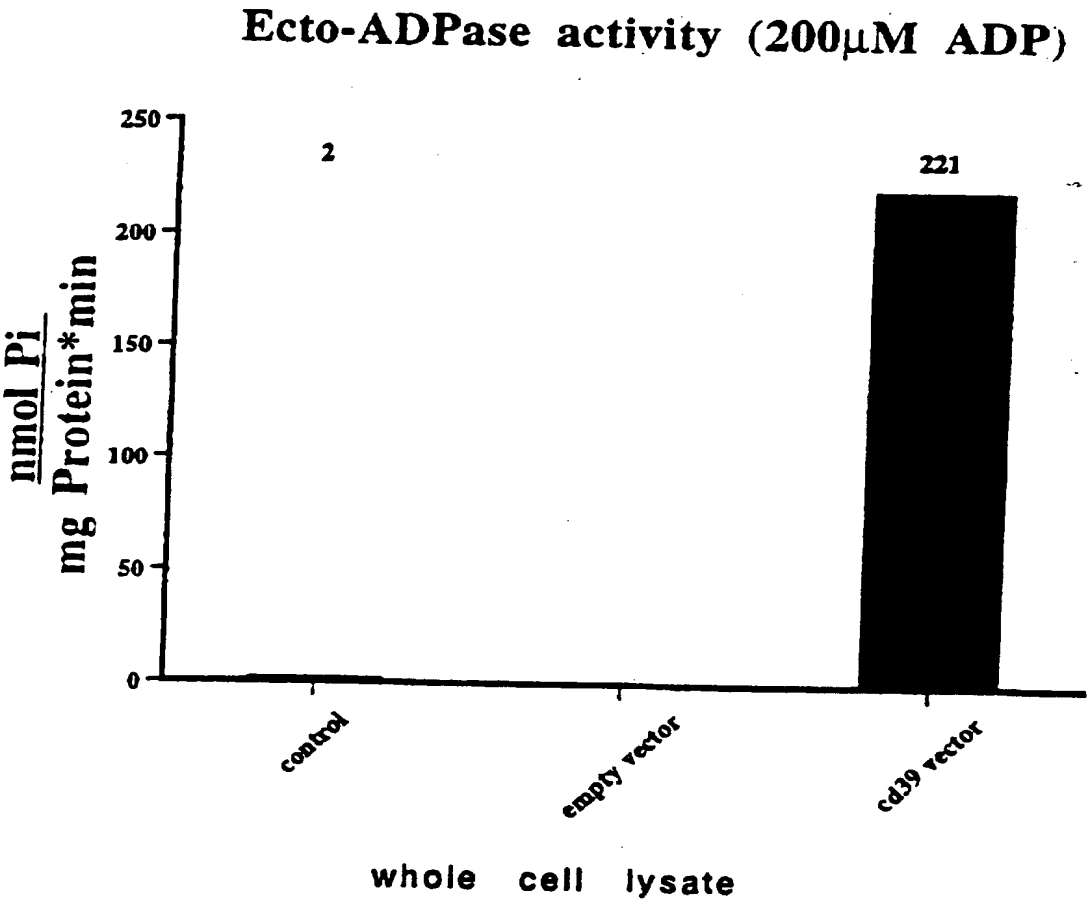


FIG. 12

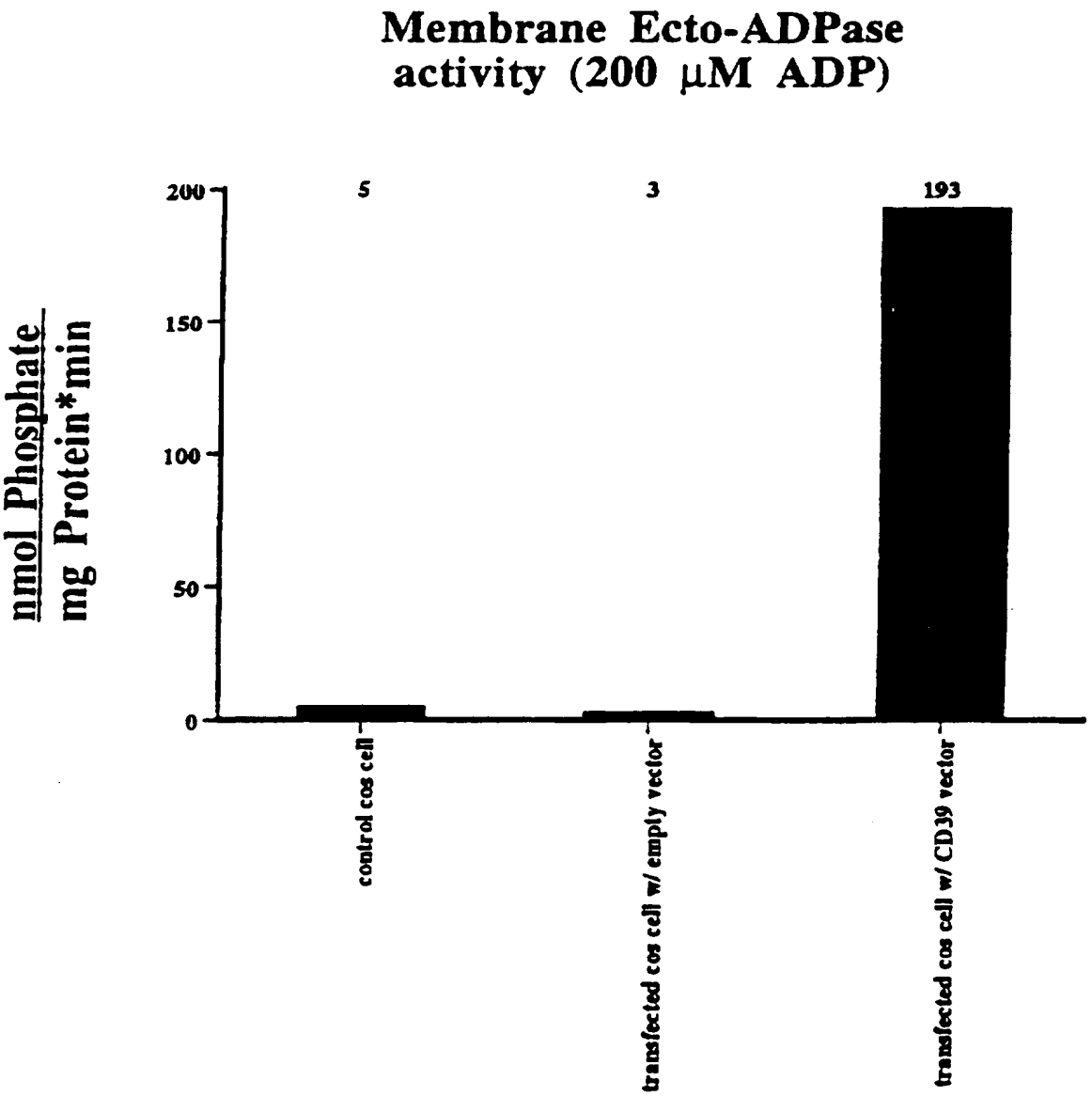


FIG. 13

Aggregation of PRP with 5 μM ADP and cos-7 cell membrane extracts (27.4 μg Protein)

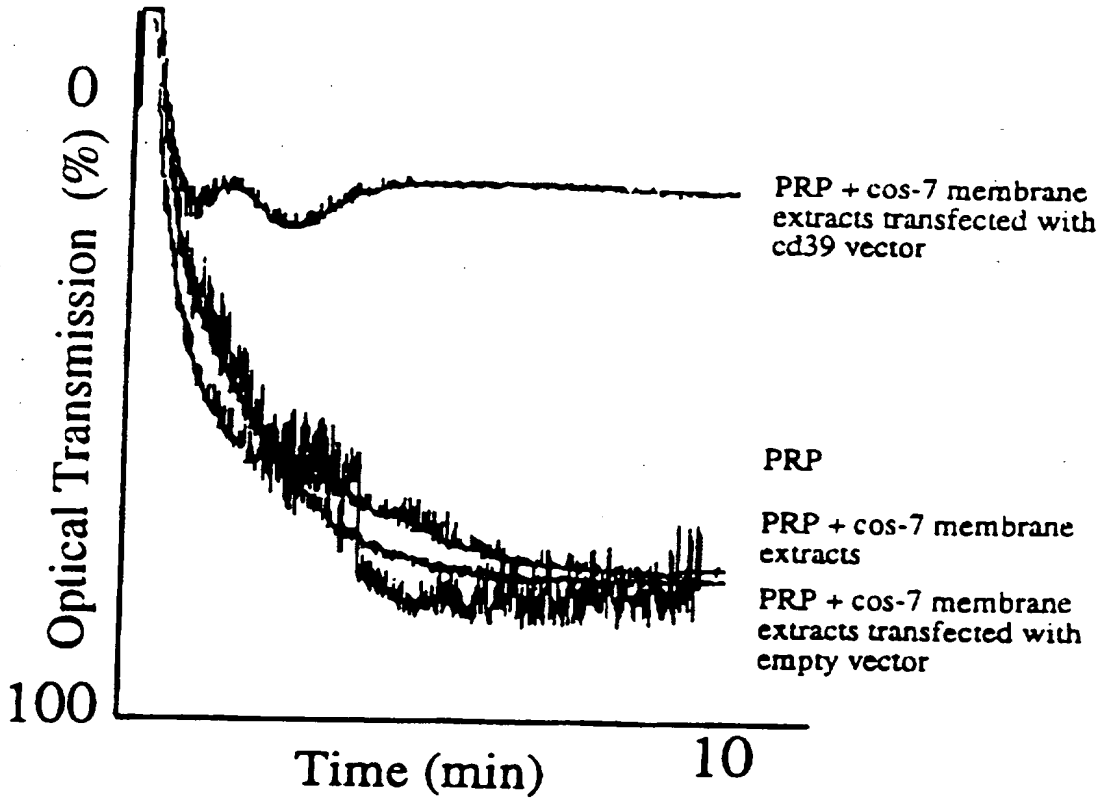


FIG. 14 (beginning)

```
   1  ACCACACCAAGCAGCGGCTGGGGGGGGGAAAGACGAGGAAAGAGGAGGAAAACAAAAGCTGCTACTTATGGAAGATACAAAGGAGTCTAACGTGAAGACA
   1                                                                                  M  E  D  T  K  E  S  N  V  K  T

 101  TTTTGCTCCAAGAATATCCTAGCCATCCTTGGCTTCTCCTCTATCATAGCTGTGATAGCTTTGCTTGCTGTGGGGTTGACCCAGAACAAAGCATTGCCAG
  12  F  C  S  K  N  I  L  A  I  L  G  F  S  S  I  I  A  V  I  A  L  L  A  V  G  L  T  Q  N  K  A  L  P  E

 201  AAAACGTTAAGTATGGGATTGTGCTGGATGCGGGTTCTTCTCACACAAGTTTATACATCTATAAGTGGCCAGCAGAAAAGGAGAATGACACAGGCGTGGT
  46    N  V  K  Y  G  I  V  L  D  A  G  S  S  H  T  S  L  Y  I  Y  K  W  P  A  E  K  E  N  D  T  G  V  V

 301  GCATCAAGTAGAAGAATGCAGGGTTAAAGGTCCTGGAATCTCAAAATTTGTTCAGAAAGTAAATGAAATAGGCATTTACCTGACTGATTGCATGGAAAGA
  79    H  Q  V  E  E  C  R  V  K  G  P  G  I  S  K  F  V  Q  K  V  N  E  I  G  I  Y  L  T  D  C  M  E  R

 401  GCTAGGGAAGTGATTCCAAGGTCCCAGCACCAAGAGACACCCGTTTACCTGGGAGCCACGGCAGGCATGCGGTTGCTCAGGATGGAAAGTGAAGAGTTGG
 112  A  R  E  V  I  P  R  S  Q  H  Q  E  T  P  V  Y  L  G  A  T  A  G  M  R  L  L  R  M  E  S  E  E  L  A

 501  CAGACAGGGTTCTGGATGTGGTGGAGAGGAGCCTCAGCAACTACCCCTTTGACTTCCAGGGTGCCAGGATCATTACTGGCCAAGAGGAAGGTGCCTATGG
 146    D  R  V  L  D  V  V  E  R  S  L  S  N  Y  P  F  D  F  Q  G  A  R  I  I  T  G  Q  E  E  G  A  Y  G

 601  CTGGATTACTATCAACTATCTGCTGGGCAAATTCAGTCAGAAAACAAGGTGGTTCAGCATAGTCCCATATGAAACCAATAATCAGGAAACCTTTGGAGCT
 179  W  I  T  I  N  Y  L  L  G  K  F  S  Q  K  T  R  W  F  S  I  V  P  Y  E  T  N  N  Q  E  T  F  G  A

 701  TTGGACCTTGGGGGAGCCTCTACACAAGTCACTTTTGTACCCCAAAACCAGACTATCGAGTCCCCAGATAATGCTCTGCAATTTCGCCTCTATGGCAAGG
 212  L  D  L  G  G  A  S  T  Q  V  T  F  V  P  Q  N  Q  T  I  E  S  P  D  N  A  L  Q  F  R  L  Y  G  K  D

 801  ACTACAATGTCTACACACATAGCTTCTTGTGCTATGGGAAGGATCAGGCACTCTGGCAGAAACTGGCCAAGGACATTCAGGTTGCAAGTAATGAAATTCT
 246    Y  N  V  Y  T  H  S  F  L  C  Y  G  K  D  Q  A  L  W  Q  K  L  A  K  D  I  Q  V  A  S  N  E  I  L
```

FIG. 14 (end)

```
 901  CAGGGACCCATGCTTTCATCCTGGATATAAGAAGGTAGTGAACGTAAGTGACCTTTACAAGACCCCCTGCACCAAGAGATTTGAGATGACTCTTCCATTC
 279   R  D  P  C  F  H  P  G  Y  K  K  V  V  N  V  S  D  L  Y  K  T  P  C  T  K  R  F  E  M  T  L  P  F

1001  CAGCAGTTTGAAATCCAGGGTATTGGAAACTATCAACAATGCCATCAAAGCATCCTGGAGCTCTTCAACACCAGTTACTGCCCTTACTCCCAGTGTGCCT
 312   Q  Q  F  E  I  Q  G  I  G  N  Y  Q  Q  C  H  Q  S  I  L  E  L  F  N  T  S  Y  C  P  Y  S  Q  C  A  F

1101  TCAATGGGATTTTCTTGCCACCACTCCAGGGGGATTTTGGGGCATTTTCAGCTTTTTACTTTGTGATGAAGTTTTTAAACTTGACATCAGAGAAAGTCTC
 346    N  G  I  F  L  P  P  L  Q  G  D  F  G  A  F  S  A  F  Y  F  V  M  K  F  L  N  L  T  S  E  K  V  S

1201  TCAGGAAAAGGTGACTGAGATGATGAAAAAGTTCTGTGCTCAGCCTTGGGAGGAGATAAAAACATCTTACGCTGGAGTAAAGGAGAAGTACCTGAGTGAA
 379   Q  E  K  V  T  E  M  M  K  K  F  C  A  Q  P  W  E  E  I  K  T  S  Y  A  G  V  K  E  K  Y  L  S  E

1301  TACTGCTTTTCTGGTACCTACATTCTCTCCCTCCTTCTGCAAGGCTATCATTTCACAGCTGATTCCTGGGAGCACATCCATTTCATTGGCAAGATCCAGG
 412   Y  C  F  S  G  T  Y  I  L  S  L  L  L  Q  G  Y  H  F  T  A  D  S  W  E  H  I  H  F  I  G  K  I  Q  G

1401  GCAGCGACGCCGGCTGGACTTTGGGCTACATGCTGAACCTGACCAACATGATCCCAGCTGAGCAACCATTGTCCACACCTCTCTCCCACTCCACCTATGT
 446    S  D  A  G  W  T  L  G  Y  M  L  N  L  T  N  M  I  P  A  E  Q  P  L  S  T  P  L  S  H  S  T  Y  V

1501  CTTCCTCATGGTTCTATTCTCCCTGGTCCTTTTCACAGTGGCCATCATAGGCTTGCTTATCTTTCACAAGCCTTCATATTTCTGGAAAGATATGGTATAG
 479   F  L  M  V  L  F  S  L  V  L  F  T  V  A  I  I  G  L  L  I  F  H  K  P  S  Y  F  W  K  D  M  V  *

1601  CAAAAGCAGCTGAAATATGCTGGCTGGAGTGAGGAAAAAATCGTCCAGGGAGCATTTTCCTCCATCGCAGTGTTCAAGGCCATCCTTCCCTGTCTGCCAG

1701  GGCCAGTCTTGACGAGTGTGAAGCTTCCTTGGCTTTTACTGAAGCCTTTCTTTTGGAGGTATTCAATATCCTTTGCCTCAAGGACTTCGGCAGATACTGT

1801  CTCTTTCATGAGTTTTTC
```

INT ATIONAL SEARCH REPORT

International Application No
PCT/EP 96/01270

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 A01K67/027 C12N5/10 C12N15/55 A61K38/46
A61K48/00 A61L33/00 //C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K A01K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 02015 (MONTEFIORE MEDICAL CENTER) 3 February 1994 see examples see claims ---	14,15, 37-41
Y	TEXAS HEART INSTITUTE JOURNAL, vol. 21, no. 1, 1994, HOUSTON, TX, USA, pages 98-103, XP000576017 K. WU ET AL.: "Gene therapy for vascular diseases." see page 98, line 1 - line 7 see page 98, line 14 - line 16 see page 102, left-hand column, line 20 - right-hand column, line 12 ---	1-13, 17-33
Y	WO,A,94 10305 (SANDOZ LTD.) 11 May 1994 see the whole document --- -/--	1-13, 17-33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 July 1996	25. 07. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF IMMUNOLOGY, vol. 153, no. 8, 15 October 1994, BALTIMORE, MD, USA, pages 3574-3583, XP002008568 C. MALISZEWSKI ET AL.: "The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization." cited in the application see abstract see figures 2,7 ---	39
A	THE JOURNAL OF IMMUNOLOGY, vol. 146, no. 7, 1 April 1991, BALTIMORE, MD, USA, pages 2235-2244, XP002008569 G. KANSAS ET AL.: "Expression, distribution, and biochemistry of human CD39. Role in activation-associated homotypic adhesion of lymphocytes." cited in the application see abstract ---	39
A	INTERNATIONAL JOURNAL OF BIOCHEMISTRY, vol. 26, no. 3, March 1994, OXFORD, GB, pages 437-448, XP000575997 A. KETTLUN ET AL.: "Human placental ATP-diphosphohydrolase: biochemical characterization, regulation and function." see abstract ---	39,40
A	SEMINARS IN HEMATOLOGY, vol. 31, no. 4, October 1994, NEW YORK, NY, USA, pages 261-269, XP000575996 A. MARCUS: "Thrombosis and inflammation as multicellular processes: Significance of cell-cell interactions." see page 264, right-hand column, line 14 - page 265, left-hand column, line 40 see 'future perspectives' ---	34-39
A	WO,A,94 12211 (AKTIBOLAGET ASTRA) 9 June 1994 see claims 1-3,13-17,20,21 see examples -----	11,12, 14,15, 17-19, 37-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 96/01270

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 22-36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/01270

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9402015	03-02-94	US-A-	5378601	03-01-95
		AU-B-	4665393	14-02-94
WO-A-9410305	11-05-94	AU-B-	5440494	24-05-94
		CA-A-	2143489	11-05-94
		EP-A-	0669977	06-09-95
		FI-A-	952088	02-05-95
		JP-T-	8502653	26-03-96
		NO-A-	951628	03-07-95
WO-A-9412211	09-06-94	AU-B-	5582194	22-06-94
		EP-A-	0668780	30-08-95
		JP-T-	8503609	23-04-96